Molekulare Mechanismen der Kolonkrebsprävention: Untersuchungen zum Einfluss von Darmfermentationsprodukten auf Entgiftungsenzyme nicht-transformierter Kolonzellen

DISSERTATION

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Abkürzungsverzeichnis

ACF	Aberrante Kryptfoci
AICR	American Institute for Cancer Research
AOM	Azoxymethan
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
ARE	Antioxidant response element
bp	Basenpaare
CDNB	1-Chloro-2,4-dinitrobenzene
COX	Cyclooxygenase
DCC	Deleted in colon cancer
DNA	Desoxyribonukleinsäure
EPIC	European Perspective Investigation into Cancer and Nutrition
FAP	Familiäre adenomatöse Polypose
GSH	Glutathion
GSR	Glutathionreduktase
GST	Glutathion-*S*-Transferase
H_2O	Wasser
H_2O_2	Wasserstoffperoxid
HNE	4-Hydroxy-2-nonenal
HNPCC	Hereditäres nicht-polypöses Kolonkarzinom
iNOS	Induzierbare Stickstoffmonoxid -Synthase
k-ras	Kirsten rat sarcoma
MAPK	Mitogen-activated protein kinase
NO	Stickstoffmonoxid
Nrf2	Nuclear factor E2-related factor 2
ROS	Reaktive Sauerstoffspezies
SOD	Superoxid-Dismutase
Sp-1	Specific protein-1
TP53	Tumorprotein 53
WCRF	World Cancer Research Fund

1 Einleitung

In Deutschland erkranken jährlich etwa 425 000 Menschen an Krebs. Über 200 000 Personen sterben an den Folgen einer Krebserkrankung (Statistisches Bundesamt, 2002). Bösartige Neubildungen stellen mit diesen Zahlen für Männer und Frauen nach Herz-Kreislauf-Erkrankungen die zweithäufigste Todesursache dar. Für Krebsneuerkrankungen und Krebssterbefälle steht Darmkrebs für beide Geschlechter an zweiter Stelle. Die Entstehung einer Krebskrankheit ist nicht auf eine Ursache, sondern auf das Zusammenspiel verschiedener Faktoren zurückzuführen. Als vermeidbare Risikofaktoren kommen dem Zigarettenrauchen und einer falschen Ernährungsweise eine besondere Bedeutung zu (Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. & Robert Koch Institut, 2006).

1.1 Ernährung und Kolonkrebs

Die menschliche Ernährung liefert einerseits zahlreiche nutritive, lebensnotwendige Inhaltsstoffe. Zusätzlich werden weitere Inhaltsstoffe verzehrt, die einen positiven Einfluss auf die Gesundheit haben können. Andererseits werden auch potentiell schädliche Substanzen aufgenommen (Berlau *et al.*, 2004). So ist die Ernährungsweise der westlichen Industrienationen durch einen hohen Verzehr an Fleisch, Fleischwaren und tierischen Fetten bei einer eher geringen Aufnahme an Obst, Gemüse und Vollkornprodukten geprägt. Gleichzeitig ist die Inzidenz für ernährungsabhängige Erkrankungen vergleichsweise höher als in Entwicklungsländern (World Cancer Research Fund, 1997). Laut World Cancer Research Fund (WCRF) könnten bis zu 75 % der Todesfälle durch eine veränderte Ernährungsweise verhindert werden. Diese Befunde wurden durch epidemiologische Studien gestützt, welche eine positive Assoziation zwischen einer „Western Style Diet“ und erhöhter Krebsinzidenz zeigen (Willett, 1995). Die Hypothese, dass die Ernährung ursächlich und präventiv eine Rolle bei der Krebsentstehung spielt, wird trotz epidemiologischer Befunde, die keinen Zusammenhang finden (Flood *et al.*, 2002; Hung *et al.*, 2004), durch andere epidemiologische Befunde (Bingham *et al.*, 2003; Michels *et al.*, 2006) und experimentelle Daten gestützt (Wargovich *et al.*, 2000; Verghese *et al.*, 2002).

Die häufigste Ursache für die Ausbildung von Dickdarmtumoren stellen demnach individuelle „Life-Style Faktoren“ dar. Während für familiär bedingte Erkrankungen, wie dem hereditären nicht-polypösen Kolonkarzinom (HNPCC) und der familiären

adenomatösen Polypose (FAP), genetische Veränderungen verantwortlich sind, treten mehr als 70 % der Dickdarmkarzinome sporadisch auf (Abb.1). Bereits 1981 wurde beschrieben, dass im Mittel ein Drittel aller Krebserkrankungen durch die Ernährung beeinflussbar oder sogar vermeidbar wäre (Doll & Peto, 1981; Willett, 1995). Insbesondere für Dickdarmkrebs wurde ein starker Zusammenhang mit der Ernährungsweise gesehen.

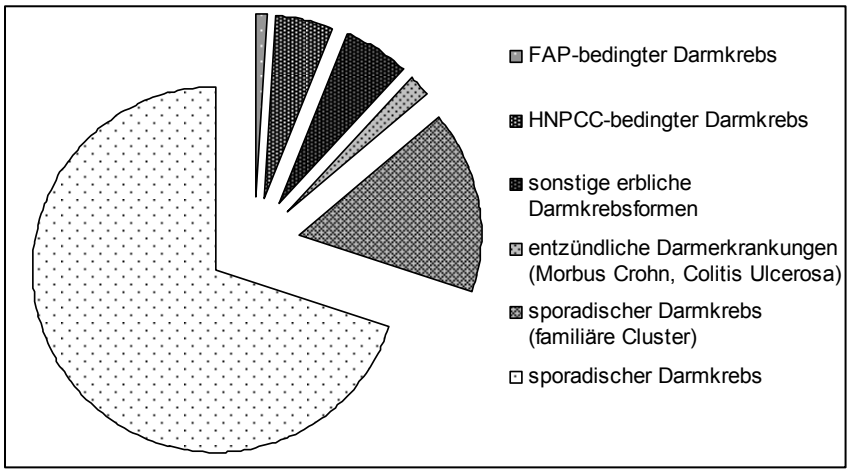

Abb. 1: Prozentuale Verteilung der Darmkrebsursachen [modifiziert nach (Potter, 1999b)].

Neueren epidemiologischen Auswertungen zu Folge wurde der präventive Effekt durch Obst- und Gemüseverzehr je nach Krebsart nur noch mit 5-12 % ausgewiesen, wobei der Einfluss der Ernährung stark von der Krebsart abhängt (Vainio & Weiderpass, 2006). Eine Neuauswertung der Datenlage wird im Frühjahr 2007 vom WCRF veröffentlicht werden.

1.1.1 Adenom-Karzinom-Sequenz

Die Tumorentstehung ist ein langjähriger multifaktorieller Prozess, in dem sich über mehrere Stufen normale Zellen zu Krebszellen entwickeln. Speziell für die Kolonkarzinogenese wurde ein Modell erstellt, in dem genetische Veränderungen mit dem Entartungsprozess in Zusammenhang gebracht wurden (Fearon & Vogelstein, 1990). Mit zunehmendem Alter steigt die Häufigkeit präneoplastischer Läsionen (Ransohoff & Lang, 1990; Paganelli *et al.*, 1990). Schätzungen zu Folge können im Alter ≥70 bei jedem zweiten Menschen, der eine „Western Style Diet“ verzehrt, Kolonadenome detektiert werden (Shpitz *et al.*, 1998; Ponz de & Roncucci, 2000; Fodde *et al.*, 2001).

Als erster Schritt der Karzinogenese auf molekularer Ebene findet die ***Initiation*** einer normalen Zelle statt, die durch eine Mutation zumeist in Protoonkogenen oder Tumorsuppressorgenen bedingt ist (Arends, 2000). Diese durch exo- oder endogene

Faktoren verursachte, nicht reversible Mutation wird an nachfolgende Tochtergenerationen weitergegeben, wodurch diese manifest wird. Eine entartete Zelle kann sich der Kontrolle des regulären Zellzyklus entziehen und hyperproliferieren. In dieser ***Promotion***sphase bilden sich zunächst gutartige Läsionen oder Präneoplasien aus. Finden in präneoplastischen Läsionen weitere Mutationen in tumor-relevanten Genen statt, entwickeln sich Adenome und daraus Karzinome (***Progression***). Diese zellulären Veränderungen zeichnen sich durch vermehrte Proliferation, verminderte Ausdifferenzierung und mangelnde Apoptose aus. Durch zunehmende genomische Instabilität und zunehmender Autonomie kann sich ein invasiver Tumor mit der Fähigkeit zur ***Metastasierung*** ausbilden (Mendelsohn, 2001).

1.1.2 Genetische Veränderungen während der Kolonkarzinogenese

Die genetischen Veränderungen im Mehrstufenmodell der Kolonkarzinogenese (Abb. 2) ereignen sich in Genen, die für Zellwachstum, Zelldifferenzierung, DNA-Reparatur und Interaktionen zwischen zellulärer und extrazellulärer Matrix verantwortlich sind (Arends, 2000).

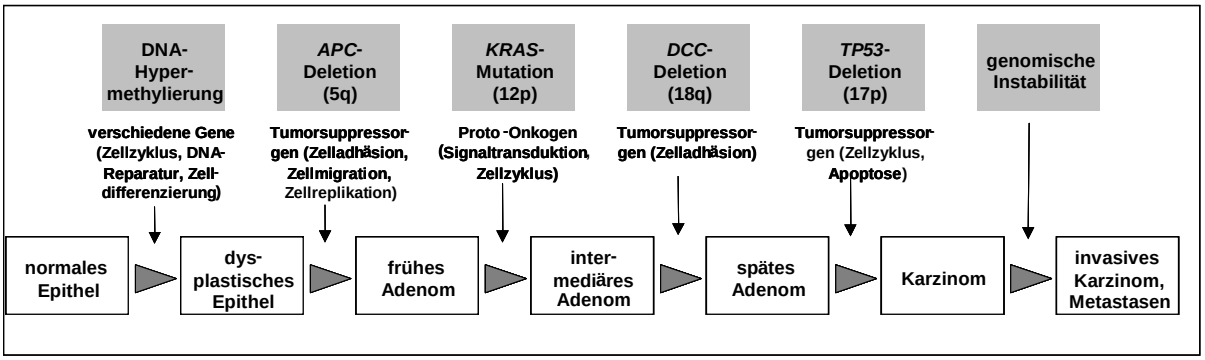

Abb. 2: Modell der Kolonkarzinogenese. Es zeigt die genetischen Veränderungen des Transformationsprozesses (Fearon & Vogelstein, 1990).

Von zentraler Bedeutung für den Entartungsprozess sind Mutationen in den Tumorsuppressorgenen APC (adenomatous polyposis coli), DCC (deleted in colon cancer) und TP53 sowie im Protoonkogen k-ras (Fearon & Vogelstein, 1990). Die Deletion und damit der Funktionsverlust des Tumorsuppressorgens APC stellt häufig die erste molekulare Veränderung dar. Durch eine Mutation im APC kommt es zu einer Akkumulation von β-Catenin im Cytoplasma, wodurch die Zellproliferation erhöht und Apoptose verringert wird. Das Protein, für das k-ras kodiert, spielt in der Signaltransduktion von Wachstumsfaktoren und damit in der Zellproliferation eine

Rolle. Schließlich führt eine Mutation im Zellzyklusregulator TP53 dazu, dass die Zellproliferation beim Auftreten von DNA-Schäden nicht blockiert wird und in den Zellen bei unzureichender Reparatur keine Apoptose ausgelöst werden kann (Fearon & Vogelstein, 1990; Arends, 2000).

1.2 Mechanismen der Chemoprävention

Der Entstehung von Krebserkrankungen kann nicht nur durch Vermeidung der Exposition mit karzinogenen Stoffen vorgebeugt werden, sondern auch durch eine gesteigerte Aufnahme protektiver Substanzen. Anhand des Modells der Kolonkarzinogenese (Abb. 2) lassen sich Ansatzpunkte ableiten, wie über ernährungsabhängige Maßnahmen präventiv in diesen Prozess eingegriffen werden kann. Diese Interventionen sollen entweder die Initiation gesunder Zellen blockieren und/oder eine weitere Promotion entarteter Zellen vermeiden.

Die Ansatzpunkte der ***Primärprävention*** stellen normale, nicht initiierte Zellen dar (Tab. 1). Eine Initiation kann durch sogenannte „Blocking Agents“ vermieden werden, indem z.B. eine Schädigung der DNA verhindert wird. Darüber hinaus können „Blocking Agents“ antioxidative Eigenschaften aufweisen, reaktive Intermediate abfangen oder die Reparatur einer bereits eingetretenen DNA-Schädigung fördern (Wattenberg, 1985).

Existieren bereits entartete Zellen oder präneoplastische Läsionen, kann deren Zellwachstum und Überleben durch sogenannte „Suppressing Agents“ eingeschränkt werden. Dies sind Mechanismen der ***Sekundärprävention*** (Tab. 1).

Tab. 1: Mechanismen der Chemoprävention [in Anlehnung an (Wattenberg, 1996)].

Primärprävention	Sekundärprävention	Therapie
- Reduktion der Toxizität - antioxidative Effekte - Induktion der Entgiftung reaktiver Substanzen - Abfangen reaktiver Intermediate - Prävention der Bildung reaktiver Intermediate - Modulation der DNA-Reparatur	- Inhibition der Proliferation - antioxidative Effekte - Induktion von Differenzierung & Apoptose - Beeinflussung der Signaltransduktion	- Inhibition der Proliferation - Apoptoseinduktion - Inhibition der Metastasenbildung - Modulation der Differenzierung - Modulation von Membran-rezeptoren, Transportsystemen - Modulation der Signaltransduktion - Immunmodulation
„Blocking Activities“		**„Suppressing Activities“**

Um Nahrungsinhaltsstoffe auf derartige Effekte hin zu untersuchen, sind menschliche Kolonzellen als *in vitro* Modelle von großer Bedeutung. Werden entartete Zellen für Untersuchungen eingesetzt, können Mechanismen der Sekundärprävention beleuchtet werden. Derartige Kolonkarzinomzelllinien sind einfach kultivierbar und zu vervielfältigen. Die Studien an Tumorzellen lassen jedoch nur begrenzte Rückschlüsse auf Mechanismen in gesunden, nicht-transformierten Zellen zu. Um den Einfluss einer Intervention am Menschen zu erfassen, werden Biomarker benötigt, die als Surrogat-Marker für das Krebsrisiko verwendet werden können. Hierfür eignen sich das Wiederauftreten von kolorektalen Adenomen oder die Detektion von präneoplastischen Läsionen, Polypen oder aberranten Kryptfoci (ACF) (Rafter *et al.*, 2004).

Als Zellmodelle für die *in vitro* Primärprävention eignen sich primäre, nicht transformierte Zellen. In diesen Zellen gilt es zu untersuchen, ob bestimmte Nahrungskomponenten protektive Effekte auf die Zellen haben und diese so vor einer Entartung schützen könnten. Für solche Untersuchungen müssen primäre Kolonepithelzellen aus humanem Kolongewebe isoliert werden (Schäferhenrich *et al.*, 2003). Von Vorteil ist dabei, dass auf diese Weise Untersuchungen an „gesunden“ Zellen durchgeführt werden können. Einen Nachteil stellen die begrenzte Verfügbarkeit des Kolongewebes sowie die begrenzte Lebenszeit nicht-transformierter Zellen dar. Experimente an primären Kolonozyten könnten jedoch dazu dienen, Mechanismen der Chemoprävention in gesunden Zellen zu beleuchten, die sich durchaus von Effekten in Tumorzellen unterscheiden können (Comalada *et al.*, 2006). Derartige Untersuchungen an nicht-transformierten Zellen sind für die Klärung der zugrunde liegenden Mechanismen der Chemoprävention von Kolonkrebs von besonderer Bedeutung.

1.2.1 Primäre humane Kolonzellen

Das Darmlumen des Menschen ist mit einer Epithelschicht ausgekleidet, die durch Kolonkrypten gekennzeichnet ist. Die Epithelschicht wird kontinuierlich erneuert, wobei Zellverlust und Zellerneuerung im Gleichgewicht stehen. An der Kryptbasis befinden sich die so genannten Kolonstammzellen, von denen eine regulierte Zellreplikation ausgeht. Diese teilungsfähigen Stammzellen sind etwa im unteren Drittel der Krypten lokalisiert, so dass sie kaum mit Inhaltsstoffen des Dickdarmlumens in Kontakt kommen. Auch eine kontinuierliche Schleimbildung schützt die Zellen (Potter, 1999b).

Stammzellen sind undifferenzierte Zellen, deren Teilung eine Stammzelle und eine Tochterzelle hervorbringt. Da die Epithelzellen ständig an der Oberfläche abgeschilfert werden, werden diese von der Kryptbasis her ersetzt. Auf dem Weg der Tochterzellen zum Darmlumen teilen sie sich weiter und differenzieren sich aus (Potten & Loeffler, 1990). Die Homöostase der Stammzellen ist von besonderer Bedeutung, da ein Verlust der Kontrolle über die Zellteilung zur Karzinogenese führen kann.

Primäre humane Kolonzellen können aus Geweberesektaten isoliert werden, die im Rahmen einer Dickdarmoperation entnommen werden. Für die in dieser Arbeit dargestellten Experimente wurde das Gewebe von Patienten verwendet, die aufgrund von Adenomen, Karzinomen oder Divertikulose operiert wurden. Um primäre Kolonzellen als *in vitro* Modelle für mechanistische Untersuchungen zu benutzen, wurden zahlreiche Versuche unternommen, diese in Kultur zu bringen.

1.2.2 Kurzzeitkulturversuche von primären Kolonzellen

Für nicht-transformierte Zellen ist es besonders problematisch, Bedingungen zu definieren, unter denen diese optimal kultiviert werden können. Eine Arbeitsgruppe setzte dafür den Zellen einen Überstand aus der Zellkultur zu, der durch vorhergehende Inkubation der Kolonepithelzellen generiert wurde und gewebespezifische Wachstumsfaktoren enthalten sollte (Panja, 2000). Diese Methode ist jedoch als kritisch zu betrachten, da Kolonepithel mit Darmbakterien besiedelt ist, welche zu einer schnellen Kontamination des Mediums führen können. Für primäre Kolonzellen ist weiterhin der Kontakt zu einer extrazellulären Matrix von großer Bedeutung, da die zumeist enzymatische Isolation aus dem Gewebeverband schnell zu Apoptose und Anoikis führt (Pedersen *et al.*, 2000; Grossmann *et al.*, 2003). Um den Kontaktverlust zur extrazellulären Matrix auszugleichen, können primäre Kolonzellen auf Kollagen-beschichtete Zellkulturgefäße ausgesät werden (Rogler *et al.*, 1998). Des Weiteren wurde das in Kulturbringen von intakten Kolonkrypten erprobt (Strater *et al.*, 1996).

Kurzzeitkulturen von primären Kolonzellen wurden in den letzten Jahren mehrfach in der Literatur beschrieben, jedoch wurde nie eine Kultivierbarkeit über wenige Tage hinaus erreicht (Rogler *et al.*, 1998; Pedersen *et al.*, 2000; Grossmann *et al.*, 2003). Die *ex vivo* Kultur von nicht-transformierten, primären Kolonzellen bleibt daher nach wie vor eine Herausforderung.

1.3 Ernährung und Prävention von Kolonkrebs

Ernährung und Lebenswandel zählen zu den wichtigsten exogenen Risikofaktoren für die Entstehung von Dickdarmkrebs. Diese Aussage lässt sich auf zahlreiche epidemiologische Studien stützen, in denen eine erhöhte Krebsinzidenz bei Verzehr einer „Western Style Diet“ gezeigt wurde (Willett, 1995).

Vor allem der Verzehr von rotem Fleisch und die dadurch bedingte höhere Eisen-Konzentration in den Fäzes ist mit einem erhöhten Dickdarmkrebsrisiko assoziiert. Es wird vermutet, dass durch das Eisen via Fenton-Reaktion vermehrt Hydroxylradikale und oxidative DNA-Schäden hervorgerufen werden (Glei *et al.*, 2002). Es wurde auch beschrieben, dass durch das Häm-Eisen endogene *N*-Nitroso-Verbindungen in den Fäzes erhöht werden, was mit der Bildung von alkylierten DNA-Addukten wie dem *O*(6)-Carboxymethyl-Guanin in ausgeschilferten Kolonzellen positiv korrelierte (Lewin *et al.*, 2006).

Als weitere vermeidbare Risikofaktoren des Lebensstils sind Alkoholkonsum, Rauchen, mangelnde Bewegung und Übergewicht zu nennen (Potter *et al.*, 1993). Im Gegensatz dazu liefern ein hoher Obst- und Gemüseverzehr sowie eine hohe Aufnahme an Ballaststoffen protektive Faktoren (Cummings & Bingham, 1998; Potter, 1999a). Obst und Gemüse stellen für den Organismus eine Quelle für die antioxidativ wirksamen Vitamine A, C und E sowie für weitere sekundäre Pflanzenstoffe dar, denen eine antikarzinogene Wirkung zugesprochen wird. Zu diesen sekundären Pflanzenstoffen zählen z.B. Polyphenole oder Glucosinolate (Gerhäuser *et al.*, 2003). Als weitere Faktoren scheinen Folsäure, Calcium und Vitamin D einen Einfluss auf das Wachstum von Kolontumorzellen zu haben (Lamprecht & Lipkin, 2003).

1.3.1 Ballaststoffe

„Ballaststoffe“ ist eine Bezeichnung für essbares Pflanzen- und Tiermaterial, welches nicht durch Enzyme des menschlichen Verdauungstraktes gespalten werden kann und verschiedene Effekte auf den Verdauungstrakt und die Gesundheit des Körpers haben kann (Ferguson *et al.*, 2001). Ballaststoffe könnten möglicherweise über verschiedene Mechanismen das Dickdarmkrebsrisiko senken. Es wurde beschrieben, dass durch eine Erhöhung des Stuhlvolumens karzinogene Substanzen in den Fäzes verdünnt werden (Kim & Mason, 1996). Ferner binden Ballaststoffe sekundäre Gallensäuren, die als potentiell toxisch gelten (Nair, 1988; Alberts *et al.*,

2003), und entziehen diese so dem enterohepatischen Kreislauf. Insbesondere die bakterielle Fermentation im unteren Abschnitt des Dickdarms führt zur Bildung von kurzkettigen Fettsäuren (Cummings & Englyst, 1987). Durch diese wird eine Absenkung des pH-Wertes bedingt, was selektiv das Wachstum z.B. von Bifidobakterien fördert. Auch pH-abhängige Enzymaktivitäten und die Bildung sekundärer Gallensäuren durch bestimmte Bakterienpopulationen können so beeinflusst werden (Rowland *et al.*, 1998).

Der protektive Einfluss von Ballaststoffen auf das Risiko für die Entstehung von Dickdarmkrebs wird jedoch in der Literatur kontrovers diskutiert. Einige Studien konnten keinen eindeutigen Zusammenhang zwischen einer hohen Aufnahme an Obst und Gemüse (und den darin enthaltenen Ballaststoffen) und dem Risiko für Kolonkrebs zeigen (Giovannucci *et al.*, 1992; Michels *et al.*, 2005; Park *et al.*, 2005). Die Gründe dafür könnten in Limitationen der epidemiologischen Studien liegen (Schatzkin & Kipnis, 2004). Außerdem könnte in einigen Studien die Gesamtaufnahme an Ballaststoffen zu gering (<30 g/Tag) gewesen sein, als dass protektive Effekte zum Tragen kommen konnten. Zusätzlich bestimmen Qualität und Zusammensetzung der Ballaststoffe ihre Wirkungen, indem unterschiedliche Fermentationsmuster entstehen (Schwiertz *et al.*, 2002). Hierbei spielt vor allem die Konzentration der gebildeten kurzkettigen Fettsäuren eine Rolle. Als weiterer Grund für fehlende positive Effekte durch Ballaststoffe könnte eine unzureichende Aufnahme anderer Phytoprotektanten mit antioxidativen Potential sein.

Dagegen existieren eine Vielzahl von *in vitro* Untersuchungen, Tierstudien (McIntyre *et al.*, 1993) und Studien am Menschen, die einen protektiven Einfluss von Ballaststoffen aufzeigen konnten. Beispielsweise zeigte die EPIC (European Prospective Investigation into Cancer and Nutrition) Studie eine inverse Beziehung zwischen der Ballaststoffaufnahme und dem Risiko für die Entstehung von Kolonadenomen oder -karzinomen. Die Gruppe mit der höchsten Ballaststoffaufnahme (34 g/Tag) hatte ein um 40 % verringertes Risiko für Dickdarmtumore im Vergleich zur Gruppe mit geringer Ballaststoffzufuhr (12 g/Tag) (Bingham *et al.*, 2003). Auch nach Einbeziehen der Folsäureaufnahme als Kovariable blieb eine signifikante inverse Relation zwischen Ballaststoffzufuhr und Kolonkrebsrisiko bestehen (Bingham, 2006). Neben der insgesamt aufgenommenen Menge spielt die Art der Ballaststoffe eine entscheidende Rolle. Besonders die Zufuhr von Weizenkleie, Fruktooligosacchariden und resistenter Stärke scheinen

einen protektiven Einfluss auf die Kolonkarzinogenese zu haben (Cummings & Englyst, 1987; Roberfroid, 2000b).

Einige Ballaststoffe können als Präbiotika bezeichnet werden. Dies bedeutet, dass sie als unverdauliche Lebensmittelinhaltsstoffe den Menschen als Wirtsorganismus positiv beeinflussen können, indem sie die Art, das Wachstum und die Aktivität von Bakterien im Dickdarm fördern, die für die Gesundheit des „Wirtes“ förderlich sind (Roberfroid, 2000b; Wollowski *et al.*, 2001). Zu den Präbiotika mit bifidogenem Effekt gehören auch Inulin und Fruktooligosaccharide.

1.3.2 Inulin und Oligofruktose

D-Fruktooligosaccharide sind D-Fruktane, die untereinander über β-(2→1) glykosidische Bindungen verknüpft sind. Sie kommen als Inulin mit einem Polymerisationsgrad von 10 bis 65 und als Oligofruktose aus 2 bis 10 Einheiten vor. Inulin kommt hauptsächlich in Knoblauch, Artischocken, Spargel und Zwiebeln vor. Die geschätzte tägliche Aufnahme in Europa liegt zwischen 3 und 11 g (Roberfroid, 2005). Für die industrielle Gewinnung von Inulin werden Chicoréewurzeln verwendet (Roberfroid, 2000a). Inulin und Oligofruktose können von menschlichen Enzymen des Verdauungstraktes nicht hydrolysiert werden, so dass sie der Dickdarmflora als Substrat zur Verfügung stehen. Neben bifidogenen Eigenschaften werden Fruktane zu Milchsäure und kurzkettigen Fettsäuren fermentiert. Ein *in vitro* hergestellter komplexer Fermentationsüberstand inhibierte das Wachstum humaner Kolontumorzellen und modulierte Tumorprogressionsmarker in wünschenswerter Weise (Klinder *et al.*, 2004b). In Studien mit Ratten konnten ACF durch Fütterung von Inulin reduziert werden (Reddy *et al.*, 1997; Poulsen *et al.*, 2002). Des Weiteren wiesen Azoxymethan (AOM)-behandelte Tiere nach Synergy1®-Fütterung (einer Mischung aus Inulin und Oligofruktose, hergestellt von ORAFTI) eine signifikant verringerte Anzahl an Kolontumoren auf (Femia *et al.*, 2002). Die Proliferationsrate des Kolonepithels wurde gesenkt sowie die Expression von Glutathion-*S*-Transferase Pi (GSTP) und der induzierbaren NO-Synthase in Tumoren der Inulin-gefütterten Tiere. Eine erhöhte Cyclooxygenase-2 (COX-2) Expression in Tumoren konnte durch die Fütterungsintervention gesenkt werden. Durch diese Tierstudie wurde gezeigt, dass das kolonkarzinogene Potential von AOM durch Fruktane gesenkt werden kann (Femia *et al.*, 2002).

In einer 12-wöchigen, randomisierten, doppelblinden und Plazebo-kontrollierten Interventionsstudie wurde einer Gruppe von Individuen mit erhöhtem

Kolonkrebsrisiko (behandelte Polypen- und Karzinompatienten) täglich 10 g einer Inulin-Oligofruktose Mischung verabreicht. Nach der Intervention wurden Fäzes und Blutproben sowie Biopsien genommen (Van Loo *et al.*, 2005). In den Fäzes wurde die Zusammensetzung der Mikroflora untersucht, im Blut wurden Immunparameter bestimmt und in den Biopsien wurden DNA-Schäden, die Zellproliferation der Mukosa und die Genexpression untersucht. In dieser humanen Interventionsstudie resultierte die Aufnahme von Synbiotika, einer Kombination aus Pro- und Präbiotika, in einer signifikanten Reduktion der Genotoxizität des Fäzeswassers der teilnehmenden Polypen-Patienten. Für die Karzinom-Patienten konnte dies nicht gezeigt werden. Außerdem wurden in den entnommenen Biopsien DNA-Schäden durch die Synbiotika-Intervention gesenkt werden. Diese verringerten DNA-Schäden spiegeln womöglich eine geringere Exposition gegenüber genotoxischen Substanzen wider, was einer Risikoreduktion gleichkommt (Rafter *et al.*, 2006).
In einer anderen Interventionsstudie konnte gezeigt werden, dass die Aufnahme von Fruktooligosacchariden die Butyratkonzentration in den Fäzes von Kolonadenom-Patienten auf die Ausgangskonzentration gesunder Individuen erhöhte. Die Gabe von Fruktooligosacchariden konnte außerdem die Konzentration der sekundären Gallensäure Lithocholsäure senken (Boutron-Ruault *et al.*, 2005).

1.3.3 Butyrat und andere Darmfermentationsprodukte

Die Mikroflora des Dickdarms setzt sich beim Menschen aus mehreren hundert verschiedenen Bakterienstämmen zusammen (Wang & Gibson, 1993; Gibson & Wang, 1994). Zu den im Kolon vorkommenden Bakterien zählen sowohl pathogene als auch nicht-pathogene Arten, sowie Arten, z.B. Bifidobakterien, denen eine gesundheitsfördernde Wirkung zugesprochen werden kann. Ballaststoffe sind für den Menschen unverdaulich. Sie gelangen daher bis in den Dickdarm, wo sie von Bakterien als Substrat verwertet und zu den kurzkettigen Fettsäuren Essig-, Propion- und Buttersäure fermentiert werden (Cummings, 1981). Kurzkettige Fettsäuren kommen in Konzentrationen von bis zu 100 mM im Kolon vor, wobei das durchschnittliche Verhältnis von Acetat:Propionat:Butyrat 60:25:15 beträgt (Cummings & Englyst, 1987; Scheppach *et al.*, 1992). Dieses Verhältnis und die Konzentrationen sind jedoch stark von der Zusammensetzung der Darmflora sowie der Art der aufgenommenen Ballaststoffe abhängig (Cummings & Bingham, 1998). Kurzkettige Fettsäuren werden von der Kolonmukosa rasch resorbiert, wobei Butyrat bevorzugt von der Kolonmukosa als Energiequelle verwendet wird und hauptsächlich

von *Eubakterien*, *Fusobakterien* und *Clostridia*-Arten gebildet wird (Barcenilla *et al.*, 2000).

Bei ballaststoffreicher Ernährung kann der Butyratanteil auf bis zu 20 mM ansteigen (Bourquin *et al.*, 1993). Insbesondere für Butyrat konnten zahlreiche Mechanismen einer möglichen krebspräventiven Wirkung beleuchtet werden (Hassig *et al.*, 1997; Sengupta *et al.*, 2006). Butyrat wird über den Monocarboxylat-Transporter MCT1 aufgenommen und fördert das Wachstum normaler Kolonozyten (Hague *et al.*, 1997), was sich jedoch auf das physiologische Wachstumskompartiment beschränkt (Scheppach *et al.*, 1992). Im Gegensatz dazu konnten *in vitro* Studien zeigen, dass die Zellproliferation von Tumorzellen gehemmt werden konnte (Hague & Paraskeva, 1995). Des Weiteren induziert Butyrat Apoptose und Differenzierung in entarteten Zelllinien. Diese Beobachtungen werden als ***Butyrat-Paradoxon*** bezeichnet (Gibson *et al.*, 1999). Dies beinhaltet die fördernde Wirkung von Butyrat auf normale Zellen und den hemmenden Einfluss auf Tumorzellen.

Mit physiologischen Konzentrationen konnte *in vitro* gezeigt werden, dass Butyrat Kolonzellen vor H_2O_2-induzierten DNA-Schäden schützen kann (Abrahamse *et al.*, 1999; Rosignoli *et al.*, 2001). Darüber hinaus ist Butyrat ein potenter Histondeacetylasehemmer, wodurch diese kurzkettige Fettsäure einen Einfluss auf die Genexpression nehmen kann. Es wurde bereits gezeigt, dass Entgiftungsenzyme, z.B. Glutathion-*S*-Transferasen, durch eine Butyratbehandlung induziert werden können. Über diesen Mechanismus wird die Entgiftungskapazität der Zellen gestärkt, so dass endogene oder exogene Karzinogene vermehrt konjugiert und aus den Zellen ausgeschleust werden können (Ebert *et al.*, 2001; Knoll *et al.*, 2005). Neben der Modifikation von Histonen (Kiefer *et al.*, 2006) und der Stimulation der MAP-Kinase-Kaskade (Ebert *et al.*, 2001) kann die Expression von Genen über „Butyrat-Response-Elemente“ in Promotorregionen beeinflusst werden (Davie, 2003).

Die Wirkungen von Butyrat als ein besonders wichtiges Darmfermentationsprodukt wurden bereits ausführlich untersucht. Neben Butyrat wurden auch komplexe Fermentationsprodukte aus Inulin untersucht, die chemoprotektive Eigenschaften aufwiesen (Klinder *et al.*, 2004b).

1.4 Toxische Nahrungsinhaltsstoffe

Die Kolonmukosa steht in ständigem Kontakt zu den Fäzes und den darin enthaltenen Abbauprodukten endogener oder exogener Verbindungen (Owen *et al.*,

2000). Die Ernährung beeinflusst die Toxizität der Fäzes (Rieger *et al.*, 1999), was z.B. anhand der Genotoxizität des Fäzeswassers *in vitro* erfasst werden kann (Klinder *et al.*, 2004a). Einerseits kann eine „Western Style Diet“ zur Aufnahme zahlreicher karzinogener Substanzen führen. So liegt die Konzentration für heterozyklische aromatische Amine und polyzyklische aromatische Kohlenwasserstoffe in stark gebratenem Fleisch deutlich höher (Eisenbrand & Tang, 1993). Andererseits werden bei einer Antioxidantien-armen Ernährung vermehrt reaktive Sauerstoffspezies (ROS) gebildet, die mit zellulären Makromolekülen (z.B. Membranen) reagieren können (Erhardt *et al.*, 1998). Die Lipidperoxidation ist ein Prozess, bei dem mehrfach ungesättigte Fettsäuren durch ROS angegriffen werden. Ein genotoxisches Abbauprodukt stellt das 4-Hydroxy-2-nonenal (HNE) dar (Knoll *et al.*, 2005).

Auch eine hohe Eisenaufnahme kann zur Bildung von ROS führen (Erhardt *et al.*, 1998), da Eisen als Übergangsmetall mit Peroxiden, wie z.B. H_2O_2 in der Fenton-Reaktion reagieren kann. Eisen, zusammen mit H_2O_2 bzw. den resultierenden ROS kann zu Zellschädigungen einschließlich Mutationen führen (Babbs, 1990; Lund *et al.*, 1999).

Von großer Bedeutung für den Schutz der Zellen vor Noxen sind die Entgiftungsenzyme der Zelle.

1.5 Entgiftungsenzyme der Zelle

Der Fremdstoffmetabolismus der Zelle führt dazu, dass schwer ausscheidbare Xenobiotika zu polaren, besser ausscheidbaren Stoffen transformiert werden. Die ***Biotransformation*** unterteilt sich in zwei Phasen. In der ***Phase I*** werden funktionelle Gruppen durch Oxidation, Reduktion oder Hydrolyse in die auszuscheidenden Substanzen eingeführt, wodurch diese polarer werden. Durch Enzyme der ***Phase II*** werden die so aktivierten Fremdstoffe an Moleküle wie z.B. Glutathion (GSH), Sulfat oder Glucuronsäure konjugiert, was zumeist zu einer erleichterten ***Elimination*** der Endprodukte aus der Zelle führt (Marquardt & Schäfer, 1997).

Ein weiterer wichtiger Schutzmechanismus der Zellen ist die Beseitigung von ROS. Als enzymatische Schutzmechanismen katalysieren Superoxid-Dismutasen (SOD), Katalase und Peroxidasen den Abbau verschiedener reaktiver Sauerstoffspezies zu H_2O (Abb. 3).

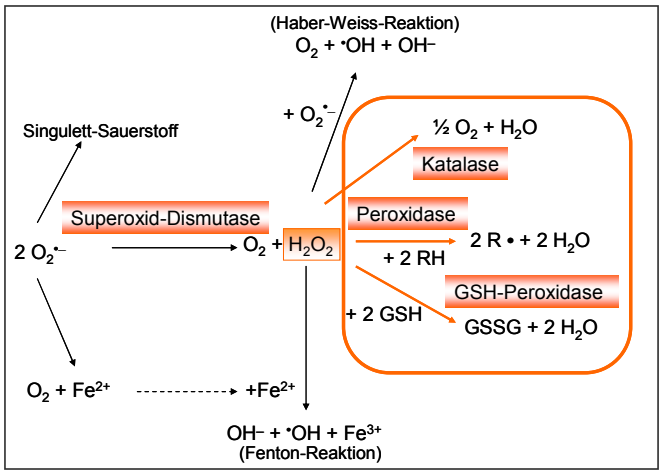

Abb. 3: Verstoffwechselung von reaktiven Sauerstoffspezies und H_2O_2 (nach Forth, Henschler, Rummel. 1998).

1.5.1 Glutathion-*S*-Transferasen

Glutathion-*S*-Transferasen (GST) sind eine Familie von Isoenzymen, die elektrophile Verbindungen mit GSH konjugieren und damit detoxifizieren. Alle eukaryotischen Organismen besitzen zytosolische und mikrosomale GST-Isoenzyme. Die humanen, zytosolischen GST werden in die Klassen Alpha, Mü, Pi, Theta, Sigma, Kappa, Zeta und Omega unterteilt. Viele GST besitzen zusätzlich eine Glutathion-peroxidaseaktivität, wodurch sie Hydroperoxide zu den entsprechenden Alkoholen reduzieren können (Eaton & Bammler, 1999). Neben diesen wichtigen detoxifizierenden Eigenschaften kann eine Überexpression in Tumoren zu einer Chemoresistenz durch Inaktivierung von therapeutisch eingesetzten Zytostatika zur Folge haben (Eaton & Bammler, 1999). Für einige Fremdstoffe wie Dichlormethan kann auch metabolische Aktivierung durch GSTT1 erfolgen, die z.B. zur DNA-Adduktbildung führen kann (Hayes & Pulford, 1995).

Für die verschiedenen Isoenzyme sind unterschiedliche Substratspezifitäten beschrieben (Hayes & Strange, 2000). GSTP1 hat eine hohe Affinität zu Benzo(a)pyren-Metaboliten, GSTM2 zu Aminochromen oder 1,2-Dichlor-4-Nitrobenzol. Der GSTA4 kommt eine wichtige Bedeutung bei der Entgiftung des Lipidperoxidationsproduktes HNE zu. GSTT2 besitzt eine hohe Affinität zu Hydroperoxiden, da diese Isoform zusätzlich Peroxidaseaktivität besitzt (Tan *et al.*, 1996). GST kann darüber hinaus eine Rolle bei zellulären Transport- oder Signaltransduktionsprozessen zugesprochen werden (Hayes *et al.*, 2005).

Für einige GST wurden Polymorphismen beschrieben. Wenn diese Polymorphismen katalytisch relevante Regionen betreffen, kann die Enzymaktivität und damit die Entgiftungskapazität beeinträchtigt werden. Deletionspolymorphismen sind für GSTT1 und GSTM1 bekannt (Eaton & Bammler, 1999), eine Basenpaar-Deletion ist für einen Polymorphismus der GSTM3 verantwortlich und eine Transitionsmutation führt zu einem Aminosäureaustausch der GSTP1 (Eaton & Bammler, 1999; McIlwain *et al.*, 2006). Neben Geschlecht, Alter und modulierenden Einflussfaktoren wird die GST-Expression auch vom Genotyp bestimmt (Coles *et al.*, 2000). Da die Expression von GST einer starken interindividuellen Variation unterliegt (Ebert *et al.*, 2003), können große Unterschiede bezüglich der zellulären Empfindlichkeit gegenüber Xenobiotika auftreten (Pool-Zobel *et al.*, 2005b).

1.5.2 Antioxidative und inflammatorische Enzyme

Chronisch entzündliche Erkrankungen des Verdauungstraktes stellen ein erhöhtes Risiko für die Krebsentstehung dar (Itzkowitz & Yio, 2004). Die mit degenerativen Erkrankungen einhergehenden Entzündungsprozesse bedingen eine verstärkte Exposition gegenüber oxidativem Stress und den dabei entstehenden ROS, die zu molekularen Schädigungen in den betroffenen Zellen führen können (Toyokuni *et al.*, 1995). Katalase ist eines der Schlüsselenzyme der enzymatischen Abwehr gegenüber oxidativem Stress, da es sehr schnell H_2O_2 zu H_2O und O_2 entgiftet (Deisseroth & Dounce, 1970). Ein hohes Expressionsniveau kann daher mit weniger DNA-Schäden aufgrund von ROS einhergehen, was durch Verringerung der zellulären Exposition das kolorektale Krebsrisiko senken könnte. Bisher wurde gezeigt, dass die Expression von Katalase durch oxidativen Stress und H_2O_2 induzierbar ist (Shull *et al.*, 1991). Durch eine Vorbehandlung humaner Kolonzellen mit physiologischen Butyratkonzentrationen konnte eine Reduktion des genotoxischen Potentials von H_2O_2 erreicht werden (Abrahamse *et al.*, 1999; Rosignoli *et al.*, 2001). Diese Studien lassen vermuten, dass Enzyme der oxidativen Stress-Abwehr durch das Fermentationsprodukt Butyrat induzierbar sind.

Neben der Aufnahme an exogenen Antioxidantien in Obst und Gemüse bestimmen genetische Polymorphismen die Abwehrkapazität der Zellen vor oxidativem Stress. Für das Katalasegen ist ein Promotor-Polymorphismus beschrieben [-262 C→T; (Forsberg *et al.*, 2001)], der zu einer verringerten Enzymaktivität führt. Es konnte ferner gezeigt werden, dass die Enzymaktivität in Personen mit einem weniger

aktiven Genotyp durch einen hohen Verzehr an Obst und Gemüse positiv beeinflusst werden kann (Ahn *et al.*, 2006).

Die SOD katalysiert die Disproportionierung reaktiver Sauerstoffspezies wie $O_2^{\bullet-}$ zu molekularem Sauerstoff und Wasserstoffperoxid, welches kurzfristig für die Zelle ein weniger toxisches Produkt darstellt. Eine Studie mit Ratten zeigte, dass durch die Applizierung des Karzinogens Dimethylhydrazin antioxidative Enzymsysteme wie SOD, Katalase und Glutathionperoxidase reduziert wurden. Durch die Supplementation des Futters mit Ingwer wurden diese Enzymsysteme induziert (Manju & Nalini, 2005). Ähnliche Ergebnisse lieferte eine Studie nach Gabe von Kurkumin. So wurden gesteigerte Enzymaktivitäten von Glutathionperoxidase, SOD und Katalase in der Leber der Ratten gemessen (Devasena *et al.*, 2002). *In vitro* konnte gezeigt werden, dass Hämoglobin womöglich über die Bildung von ROS zu einer Steigerung der SOD1-Expression führt (Lee *et al.*, 2006). In einer Interventionsstudie konnte dagegen gezeigt werden, dass durch eine hohe Aufnahme von antioxidativ wirksamen Pflanzeninhaltsstoffen die SOD-Aktivität in Erythrozyten gesenkt wurde (Bruce *et al.*, 2000). Diese Untersuchungen zeigen, dass antioxidative Enzymsysteme zum einen durch Nahrungsinhaltsstoffe induzierbar sind, zum anderen aber auch bei ausreichender Aufnahme von nicht-enzymatischen Antioxidantien herunter reguliert werden.

Die Cyclooxygenase-2 (COX-2) wird in Kolontumoren überexprimiert (Church *et al.*, 2004), was zu einer vermehrten Bildung von inflammatorischen Prostaglandinen führt. Da inflammatorische Prozesse wiederum zu oxidativem Stress mit Bildung von freien Radikalen führen können (Wang *et al.*, 2005), stellt die Hemmung von COX-2 möglicherweise einen effektiven Mechanismus der Krebs-Chemoprävention dar (Fournier & Gordon, 2000). Ferner konnte gezeigt werden, dass COX-2-Inhibitoren die Zellproliferation in Kolonadenokarzinomzellen hemmen und Apoptose induzieren (Richter *et al.*, 2001). Darüber hinaus wurde die Anzahl AOM-induzierter ACF in Ratten durch Kombination von Fruktooligosacchariden und COX-2 Inhibitoren im Futter verringert (Buecher *et al.*, 2003).

1.5.3 Modulation der Entgiftungskapazität

Chemoprotektive Agentien der Ernährung sollten die Fähigkeit besitzen, eine mögliche Giftung in der ***Phase I*** zu hemmen, während die Aktivität entgiftender Enzyme (***Phase II***) der Zellen gesteigert wird.

GST können durch Xenobiotika über verschiedene transkriptionelle Mechanismen induziert werden (Hayes & Pulford, 1995). Eine Induktion von GST könnte zur Reduktion des Kolonkrebsrisikos beitragen, da durch verstärkte Konjugation elektrophiler Zwischenprodukte die Exposition gegenüber toxischen/genotoxischen Xenobiotika verringert werden kann.

Die DNA besitzt responsive Elemente, die mit Antioxidantien oder planaren aromatischen Kohlenwasserstoffen interagieren können. Es konnte gezeigt werden, dass GST über ein „antioxidant response element“ (ARE) in der regulatorischen Region des Gens verfügen, was beispielsweise durch phenolische Antioxidantien aktiviert werden kann (Hayes *et al.*, 2005). Auch der Transkriptionsfaktor Nrf2 kann durch Interaktion mit dem ARE die Expression von Phase II-Genen auslösen. Ferner wurden AP-1 Bindungsstellen in den Promotorregionen von GSTA1, GSTA4 und GSTP1 beschrieben (Whalen & Boyer, 1998; Pool-Zobel *et al.*, 2005a). Das Fermentationsprodukt Butyrat moduliert die GST-Expression wahrscheinlich über andere Mechanismen, wie beispielsweise durch eine Erhöhung der Histonacetylierung (Kiefer *et al.*, 2006) oder durch Aktivierung der MAP-Kinase-Signaltransduktion (Ebert *et al.*, 2001).

Die Expression von Katalase wird maßgeblich durch die Konzentration an Hydroperoxiden bestimmt. Des Weiteren wurden Sp-1 Bindungsstellen beschrieben, über die eine Modulation stattfinden könnte (Ahn *et al.*, 2006).

COX-2 wird über Cytokine, Wachstumsfaktoren, Mitogene und Onkoproteine induziert (Wang *et al.*, 2005). Die Aktivität von COX-2 kann dagegen durch unspezifische nicht-steroidale Antiphlogistika, wie Aspirin und Sulindac, gehemmt werden. Ferner wurden weitere spezifische Pharmaka, die selektiv die COX-2 hemmen, wie Celecoxib und Rofecoxib, als chemopräventive Agenzien untersucht, um die Entstehung von Kolonpolypen zu hemmen (Bertagnolli, 2003).

Die Kenntnis darüber, dass wichtige Ziel-Enzyme der Kolonkrebsprävention prinzipiell exogen modulierbar sind, stellt die Grundlage für Untersuchungen dar, Möglichkeiten einer ernährungsbedingten Prävention zu finden.

1.6 Ziele der Arbeit

Zur Untersuchung des Einflusses von Darmfermentationsprodukten auf primäre Kolonzellen wurden folgende Fragestellungen bearbeitet:

- Zunächst sollte die Sensitivität primärer Kolonzellen gegenüber einem ernährungsbedingten Risikofaktor der Kolonkarzinogenese erfasst werden. Dazu wurde die Genotoxizität von Hämoglobin und Hämin quantifiziert (mittels Comet Assay; **Publikation I**).
- Für alle Untersuchungen zur Genexpression war die Voraussetzung, eine verbesserte Primärzellkultur zu entwickeln und zu erproben. Grundlage dafür stellte eine bereits publizierte Methode dar (Rogler *et al.*, 1998).
- Mittels Gen-Arrays wurden Untersuchungen zur Basalexpression von Genen des Fremdstoffmetabolismus durchgeführt (**Publikation II**).
- In den anschließenden Versuchen wurde die Sensitivität primärer Kolonzellen gegenüber Butyrat und einem komplexen Fermentationsüberstand aus Inulin ermittelt (mittels Vitalitätsassays; **Publikationen III, IV**).
- Ferner sollten Butyrat bzw. der Fermentationsüberstand auf ihr Potential untersucht werden, verschiedene Fremdstoff-metabolisierende und oxidativer Stress-assoziierte Gene zu induzieren (mittels Gen-Arrays, real-time PCR; **Publikationen III, IV, V**).
- Darüber hinaus wurden die Konsequenzen einer veränderten Genexpression auf Proteinebene ermittelt (mittels Enzymaktivitäten von Glutathion-*S*-Transferasen und Katalase; **Publikationen III, IV, V**).
- Unter Verwendung von normalen und Tumorzellen des gleichen Spenders wurde ein Vergleich der Wirkung von Butyrat auf Zellen unterschiedlichen Transformationsgrades vorgenommen (Gen-Arrays, real-time PCR; **Manuskript VI**).

2 Publikationen*

2.1 Publikation I: HEMOGLOBIN AND HEMIN INDUCE DNA DAMAGE IN HUMAN COLON TUMOR CELLS HT29 CLONE 19A AND IN PRIMARY HUMAN COLONOCYTES.

Michael Glei, Stefanie Klenow, **Julia Sauer**, Uta Wegewitz, Konrad K. Richter, Beatrice L. Pool-Zobel. *Mutation Research*. 2006; 594 (1-2):162-71.

Epidemiologische Studien konnten zeigen, dass eine hohe Aufnahme an rotem Fleisch das Risiko für Kolonkrebs erhöht (Marchand *et al.*, 2001; Lee *et al.*, 2004). Ziel dieser Studie war es, zu untersuchen, ob das in rotem Fleisch enthaltene Hämoglobin oder seine prosthetische Gruppe Hämin einen genotoxischen Riskofaktor darstellen. Dafür wurden humane Kolonzellen mit Hämoglobin und Hämin inkubiert, woraufhin DNA-Schäden mittels Comet Assay erfasst wurden. Die hier verwendeten primären, nicht-transformierten Kolonzellen stellen relevante Target-Zellen dar, in denen genotoxische Effekte zur Krebsinitiation beitragen können.

Hämoglobin induzierte DNA-Schäden in Kolonzellen, die mit der Entstehung von freien Radikalen und zytotoxischen Effekten zusammenhängen. Durch seine zytotoxischen und genotoxischen Wirkungen stellen daher Hämoglobin oder Hämin, wie es aus rotem Fleisch verfügbar ist, einen Risikofaktor dar, der zur Initiation oder Progression der Kolonkarzinogenese beitragen kann.

Eigenanteil:

- Zellisolierung und Aufarbeitung des primären Kolongewebes aus Gewebeproben
- Durchführung, Auswertung und Darstellung der Comet Assays mit primären Kolonzellen
- Anteilige Verfassung des Manuskriptes

* Weitere Publikationen, die nicht in den engeren thematischen Zusammenhang der Dissertation gehören, erscheinen in der Publikationsliste am Ende der Arbeit. Experimentelle Arbeiten meinerseits während der Promotion führten zur Aufführung im Autorenverzeichnis.

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Hemoglobin and hemin induce DNA damage in human colon tumor cells HT29 clone 19A and in primary human colonocytes

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Abstract

Epidemiological findings have indicated that red meat increases the likelihood of colorectal cancer. Aim of this study was to investigate whether hemoglobin, or its prosthetic group heme, in red meat, is a genotoxic risk factor for cancer. Human colon tumor cells (HT29 clone 19A) and primary colonocytes were incubated with hemoglobin/hemin and DNA damage was investigated using the comet assay. Cell number, membrane damage, and metabolic activity were measured as parameters of cytotoxicity in both cell types. Effects on cell growth were determined using HT29 clone 19A cells. HT29 clone 19A cells were also used to explore possible pro-oxidative effects of hydrogen peroxide (H_2O_2) and antigenotoxic effects of the radical scavenger dimethyl sulfoxide (DMSO). Additionally we determined in HT29 clone 19A cells intracellular iron levels after incubation of with hemoglobin/hemin. We found that hemoglobin increased DNA damage in primary cells ($\geq$10 μM) and in HT29 clone 19A cells ($\geq$250 μM). Hemin was genotoxic in both cell types (500–1000 μM) with concomitant cytotoxicity, detected as membrane damage. In both cell types, hemoglobin and hemin ($\geq$100 μM) impaired metabolic activity. The growth of HT29 clone 19A cells was reduced by 50 μM hemoglobin and 10 μM hemin, indicating cytotoxicity at genotoxic concentrations. Hemoglobin or hemin did not enhance the genotoxic activity of H_2O_2 in HT29 clone 19A cells. On the contrary, DMSO reduced the genotoxicity of hemoglobin, which indicated that free radicals were scavenged by DMSO. Intracellular iron increased in hemoglobin/hemin treated HT29 clone 19A cells, reflecting a 40–50% iron uptake for each compound. In conclusion, our studies show that hemoglobin is genotoxic in human colon cells, and that this is associated with free radical mechanisms and with cytotoxicity, especially for hemin. Thus, hemoglobin/hemin, whether available from red meat or from bowel bleeding, may pose genotoxic and cytotoxic risks to human colon cells, both of which contribute to initiation and progression of colorectal carcinogenesis.

Keywords: Colon cells; HT29 clone 19A; Hemoglobin; Hemin; Single cell microgelelectrophoresis (comet assay); H_2O_2

1. Introduction

Experimental data in humans have shown that high consumption of red and processed meat and alcohol, in combination with low consumption of vegetables and lack of exercise, coupled with genetic predisposition, increase the risk of developing colorectal cancer [1–4]. Pierre et al. have reported that hemoglobin and hemin given in a low-calcium diet to rats promote colorectal carcinogenesis at the aberrant crypt stage [5]. Recent results from human studies also suggest that intake of dietary heme iron is associated with an increased risk of prox-

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imal colon cancer, especially among women who drink [6]. Related to this is at least one finding that carriers of gene mutations (C282Y or H63D) for hemochromatosis (HFE), an autosomal recessive disease associated with increased body iron stores, have a significantly increased risk for developing colon cancer [7]. In extension of this, however, another study could not find the same association for the most common germ line mutations in the HFE gene, namely C282Y or H63D, whereas, there was a trend for an association in carriers of C282Y and H63D compound heterozygosity (C282Y/wild type and H63D/wild type) [8].

Meat, alcohol and ferrous iron are suspected of increasing the formation of free radicals in the bowel, especially of reactive oxygen species [9–11]. These radicals may damage the cells of the colon crypt or enhance tumor progression [12]. We have previously shown that human colon cell lines, treated with 250 μM ferric iron nitrilotriacetate (Fe-NTA) for 15 min to 24 h rapidly absorbed iron. Moreover, Fe-NTA (250–1000 μM) induced DNA breaks and oxidized DNA bases, which were enhanced by subsequent H_2O_2 exposure [13]. We also demonstrated that hemoglobin was as effective as Fe-NTA in inducing DNA damage [13]. We had, however, not investigated whether iron from hemoglobin or from its iron-containing prosthetic group, heme, was absorbed by the colon cells, and whether the induced DNA damage was associated with the generation of free radicals. These types of qualitative and quantitative data on the genotoxic impact of iron in the gut are needed to determine risk potentials. In the present study, we therefore investigated whether hemoglobin and hemin (oxidized heme) induce DNA damage in human colon cells and whether the resulting genotoxicity is related to generation of oxidative stress and to free radical formation. In addition to using HT29 clone cells, as in our first study, here we also used primary human colon cells. These non-transformed primary cells are relevant targets for the study of colon cancer risk compounds. Such cells are especially suited to assess genotoxicity related to initiation of carcinogenesis [14].

Using HT29 clone 19A cells, we also studied some possible mechanisms of activities, such as pro-oxidative activities mediated by the physiologically abundant peroxide H_2O_2, or radical-scavenging activities mediated by the model compound DMSO, and the intracellular uptake of iron from both compounds using our in vitro culture conditions. Altogether the studies were expected to give more information on the genotoxic potential of hemoglobin, or its prosthetic group heme. Under given exposure situations (e.g. bleeding, high intake of red meat) this activity of hemoglobin is expected to contribute to initiation and progression of colorectal carcinogenesis.

2. Materials and methods

2.1. Human colon cells and in vitro conditions

HT29 clone 19A is a permanently differentiated sub-clone derived from the carcinoma cell line HT29 after sodium butyrate treatment [15]. HT29 clone 19A cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Under given laboratory conditions HT29 clone 19A cells doubled their number within 24 h. Passages 27–48 were used for the experiments.

Primary colon cells were freshly isolated from colon tissue obtained during surgical resections, as described previously [16]. The donors (*n*: nine males, age: 69 ± 9.4) of this colon tissue had given their informed consent and were admitted to the hospital for colorectal surgery. Non-tumorous tissue for cell isolation was excised together with tumor tissue for medical indications. The study was approved by the Ethical Committee of the Friedrich-Schiller-University Jena.

Bovine hemoglobin (which predominantly consists of methemoglobin since native hemoglobin is rapidly oxidized; Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in cell culture medium RPMI 1640 (Life Technologies, Karlsruhe, Germany), in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin, or minimum essential medium (MEM). The solutions in RPMI/DMEM or MEM were used for experiments with HT29 clone 19A and primary cells, respectively. Hemin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was prepared in a stock solution in 20 mM sodium hydroxide (NaOH) (Fisher Scientific, Loughborough, UK) and further diluted in the appropriate cell culture medium. Colon cells were incubated with hemin or hemoglobin at 37 °C at different concentrations (10–1000 μM) or for different periods of time as is specified in the legends of the figures and tables.

2.2. Detection of DNA damage

DNA damage was measured using single cell microgel-electrophoresis, as has been described in detail by Glei et al. [13]. Microscopical analysis revealed images of damaged DNA ("comets"). The proportion and extent of DNA migration were determined for 50 images per slide using the image analyzing system of Perceptive Instruments (Suffolk, UK, www.perceptive.co.uk). The intensity of fluorescence in the comet tail, expressed as % fluorescence in tail, was used as the evaluation criteria. For each data point mean values of three parallel slides of one experiment were the basis for calculating overall mean values of independently reproduced experiments ($n = 3$–5), as is specified in the tables and figures.

2.3. Cytotoxicity determined as metabolic activity and cell growth

The influence of hemoglobin and hemin on metabolic activity/viability and cell growth was determined in 96-well microtiter plates. Colon cells were treated with hemoglobin (0.1–1000 μM) or hemin (1–1000 μM) in culture medium for 24 and 72 h (only HT29 clone 10A cells were used for experiments lasting for 72 h). After adding 20 μl CellTiter-Blue™ (Promega, Mannheim, Germany) reagent 2 h before the end of the incubation period resarufin was converted into resazorin by metabolic active cells. The intensity of the resulting fluorescence was measured with Ex/Em 520/595 nm. In further experiments, DNA content was assessed in HT29 clone 19A cells by fixing and permeabilizing the cells with methanol for 5 min, and then adding 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) which binds to DNA. After 30 min, the DNA content per well was detected, as a reflection of the remaining cells, using fluorimetric analysis with Ex/Em 360/450 nm. Mean values (three parallel determinations per experiment, 3–6 experiments) were recorded for final evaluation.

2.4. Analysis of iron content

HT29 clone 19A cells were incubated with 250 μM hemoglobin or 32 μM hemin for a period beginning from 15 min to 24 h. The iron content of the culture medium and cells were analyzed separately. This was done using inductively coupled argon plasma emission spectrometry (Liberty Serie II ICP-AES, Varian, Darmstadt) as described previously [13].

2.5. Statistical evaluation

Data shown in the tables and figures represent mean values ± S.D. Unless otherwise stated, these mean values of at least three independent experiments were calculated from the means of triple replicates obtained in each experiment. Statistical evaluation was performed with GraphPad Prism Version 3.0 and 4.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Depending on sample size and type of experiment, *t*-test or one-way ANOVA was used to determine significance of the experimental variables. The significance of individual treatment groups in comparison to the controls was determined with the Bonferroni's multiple comparison post test (with selected pairs). EC_{50} was calculated with non-linear regression and one phase exponential decay. The statistical analyses used were dependent on the respective experimental design and are specified in the legends of the figures and tables.

3. Results

3.1. Genotoxicity

Both compounds, hemoglobin and hemin, significantly induced DNA damage in HT29 clone 19A cells with no apparent differences in the genotoxic potency of the two compounds at concentrations up to 500 μM (Table 1). At the higher concentration, hemin was cytotoxic and decreased the cell viability more in HT29 clone 19A cells than in primary colon cells. Hemin can therefore not be defined as being genotoxic at this concentra-

Table 1
Hemoglobin and hemin (15 min incubation, 37 °C) induced DNA damage (tail intensity %) in primary colon cells (unpaired *t*-test, $^{\S}p < 0.05$) and in HT29 clone 19A cells ($n = 4$–5; one-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$, Bonferroni's multiple comparison test to control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$)

Primary cells							HT29						
Fe-source [μM]	Viability [%]			Tail intensity [%]			Fe-source [μM]	Viability [%]			Tail intensity [%]		
Hemoglobin	Mean	S.D.	ns	Mean	S.D.	ns	Hemoglobin	Mean	S.D.	ns	Mean	S.D.	**
0	89.6	3.5		3.7	2.2		0	93	5		3.1	0.4	
10	93.9	2.4		11.7§	5.7		10	92	5		4	0.9	
100	86.8	8.4		13.8	7.8		100	93	4		5.5	1.6	
250	90.0	3.9		12.9§	5.9		250	94	2		7.4	1	
500	86.0	5.2		12.9§	4.9		500	92	5		9.3	0.5	*
1000	90.0	2.4		13.2§	4.9		1000	92	5		9.9	4.5	**
Hemin	Mean	S.D.	ns	Mean	S.D.	**	Hemin	Mean	S.D.	***	Mean	S.D.	***
0	90.8	2.9		3.6	1.2		0	91	5		3.4	0.8	
10	90.8	4.3		7.9	1.3		10	91	4		4	0.8	
100	92.7	2.6		7.7	2.9		100	89	8		3.5	0.3	
250	91.4	6.3		10.5	6.2		250	90	6		4.7	1.1	
500	88.8	5.4		8.4	1.8		500	70	17		10.1	5.9	
1000	86.8	8.8		12.0	4.8	*	1000	40	20	***	22.9	8.2	***

Simultaneously hemin decreased the cell viability in HT29 clone 19A (Bonferroni's multiple comparison test to control, $^{***}p < 0.001$, $n = 3$).

Table 2
Effects of hemoglobin and hemin on metabolic activity in primary cells and HT29 clone 19A cells after 24 h treatment

Hemin (μM)	Primary cells				HT29				Hemoglobin (μM)	Primary cells				HT29			
	Mean	S.D.	*n*	***	Mean	S.D.	*n*	***		Mean	S.D.	*n*	***	Mean	S.D.	*n*	***
0	100	0	4		100	0	3		0	100	0	2		100	0	3	
100	90	8	4	**	97	8	3		100	70	4	3	***	69	4	3	***
250	75	9	4	***	81	0	3	***	250	54	7	3	***	48	2	3	***
500	69	4	4	***	66	5	3	***	500	43	9	3	***	36	2	3	***
1000	51	12	4	***	47	5	3	***	1000	22	6	3	***	22	2	3	***

The activity of the medium control was set to equal 100 %. Hemoglobin and hemin significantly decreased the metabolic activity (one-way ANOVA, $^{***}p < 0.001$). Significant differences to the medium control are indicated with asterisks ($^{**}p < 0.01$, $^{***}p < 0.001$). No differences were detectable between primary cells and the carcinoma cell line.

tion (>500 μM), since false positive results (increased DNA damage) due to DNA fragmentation in dying cells cannot be excluded [17,18].

Initial DNA-damage is not different between HT29 clone 19A cells and primary colon cells. In the latter cells, hemin significantly induced DNA damage at 1000 μM whereas hemoglobin already caused DNA-damage at low concentrations (10 μM), however, without revealing a concentration-response relationship.

3.2. Metabolic activity

Metabolic activity was measured in HT29 clone 19A and in primary colon cells after 24 h incubation with hemoglobin and hemin (Table 2). Both compounds (≥100 μM) significantly reduced the metabolic activity with increasing concentrations, indicating cytotoxic effects. Both cell types were of similar sensitivity, without any significant differences between them.

3.3. Cell growth

Table 3 shows that both hemoglobin and hemin reduced the cell number of HT29 clone 19A cells after 24 and 72 h treatment. Hemin was markedly more effective than hemoglobin. The calculated EC_{50} values for 24 h of incubation were 91 μM (hemin) and not detectable for hemoglobin. After 72 h, the corresponding values were 76 μM (hemin) and 921 μM (hemoglobin). There were no significant differences between the two different durations of exposure (24 and 72 h), thus the induction of this particular cytotoxic effect did not seem to depend on time.

3.4. Iron–H_2O_2 interaction

Next, we investigated the genotoxic potentials of hemoglobin and hemin in combination with H_2O_2 to determine possible pro-oxidative activities. For this, we pre-treated (15 min, 37 °C) the HT29 clone 19A

Table 3
Effects of hemoglobin and hemin on HT29 clone 19A cell number after 24 and 72 h

Hemin (μM)	24 h				72 h				Hemoglobin (μM)	24 h				72 h			
	Mean	S.D.	*n*	***	Mean	S.D.	*n*	***		Mean	S.D.	*n*	***	Mean	S.D.	*n*	***
0	100	0	3		100	0	6		0	100	0	3		100	0	4	
1	96	4	3		111	12	6		1	97	2	3		98	13	4	
10	80	0	3	***	89	9	6		10	92	5	3		88	13	4	
50	61	2	3	***	57	7	6	***	50	90	2	3	*	81	16	4	
100	49	1	3	***	47	8	6	***	100	86	4	3	***	80	20	4	
250	33	2	3	***	28	5	6	***	250	74	4	3	***	73	17	4	*
500	25	1	3	***	20	5	6	***	500	66	4	3	***	62	11	4	**
750	19	5	3	***	18	4	6	***	750	65	4	3	***	55	9	4	***
1000	16	3	3	***	22	7	6	***	1000	54	4	3	***	46	5	4	***

The cell number of the medium control was set to equal 100%, thus each value represents the relative cell number. Hemoglobin and hemin decreased the cell number significantly (one-way ANOVA, $p < 0.001$). Significant differences to the medium control (0 μM) are indicated with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Table 4
Combination effects of hemoglobin or hemin (15 min pretreatment of cells at 37 °C) and H_2O_2 (added to cells on slides, 5 min, 4 °C) on DNA damage (tail intensity %) in HT 29 clone 19A cells

Fe-source (μM)	H_2O_2 (μM)						H_2O_2 (μM)						H_2O_2 (μM)					
	0			18.75			0			9.37			0			4.69		
Hemoglobin	Mean	S.D.		Mean	S.D.	***, ###	Mean	S.D.		Mean	S.D.	***, ###	Mean	S.D.		Mean	S.D.	###
0	2.9	0.9		10.5	2.0	***	2.5	0.2		6.9	2.2	*	3.0	0.2		6.5	0.9	
10	3.9	0.4		9.3	2.2	*	4.3	1.1		6.3	1.6		4.2	1.6		6.7	1.5	
100	7.5	3.2	#	9.4	1.3		6.2	1.5	#	7.5	1.3		6.2	2.2		8.7	0.7	
250	9.6	0.5	###	11.7	3.3		9.3	1.6	###	11.0	1.5	##	10.9	2.4	##	12.7	2.9	#
500	13.4	2.3	###	15.8	3.0	##	14.2	2.2	###	16.9	2.1	###	13.5	4.2	###	14.2	2.8	##
1000	13.2	1.0	###	18.4	0.1	*, ###	14.8	1.1	###	18.5	1.3	###	16.4	4.9	###	16.1	3.7	###
Hemin	Mean	S.D.		Mean	S.D.	***, ###	Mean	S.D.		Mean	S.D.	***, ###	Mean	S.D.		Mean	S.D.	**
0	4.1	1.0		9.1	1.8		2.4	0.4		4.6	1.4		2.5	0.2		5.3	1.6	
10	4.2	1.1		7.6	0.8		2.9	0.7		4.2	0.6		2.9	0.6		7.1	3.6	
50	5.5	0.2		9.3	2.0		3.9	0.6		7.5	1.2		4.5	2.3		8.5	3.3	
100	5.5	0.5		10.9	0.8		4.1	1.6		9.4	4.6	*, #	5.2	2.4		11.3	6.8	
250	11.1	5.1	#	16.7	4.6	#	7.2	1.5	#	8.9	1.5	#	5.9	0.9		9.8	5.8	
500	20.9	7.2	###	26.4	3.1	###	12.5	1.4	###	17.8	3.8	*, ###	9.3	3.4		11.5	5.0	

Means and S.D. from $n = 3$ independently reproduced experiments are presented. Significant differences to control without iron is indicated by #, significant differences to corresponding incubation with hemoglobin/hemin and without H_2O_2 are indicated by * (Two-way ANOVA, $^{***,###}p < 0.001$ $^{**}p < 0.01$; Bonferroni's multiple comparison test against control, $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,###}p < 0.001$).

cells in suspension with 10–1000 μM hemoglobin and 10–500 μM hemin, respectively. Afterwards, the cells (in agarose gel on slides) were damaged with low genotoxic doses of H_2O_2 (4.69–18.75 μM, 5 min, 4 °C). The combination treatment resulted in significant increases of DNA damage at only few data points (Table 4). Altogether the results merely reflected marginal additive effects by H_2O_2. Significant differences for the various treatment options were not detected.

3.5. Effects of the radical scavenger DMSO on DNA damage

DMSO is a well-known scavenger of free radicals, and thus may be used to indirectly elucidate radical mechanisms of toxicity [19]. Fig. 1 clearly demonstrates that DNA damage caused by hemoglobin and by H_2O_2 (used as a positive control on account of its radical generating properties) is clearly reduced in the presence of increasing DMSO concentrations. These results support the hypothesis that radicals formed by hemoglobin and H_2O_2 are scavenged by DMSO.

3.6. Cellular iron uptake

Iron from both hemoglobin and hemin was rapidly taken up by HT29 clone 19A cells. A significant time-effect relationship was observed. This response curve

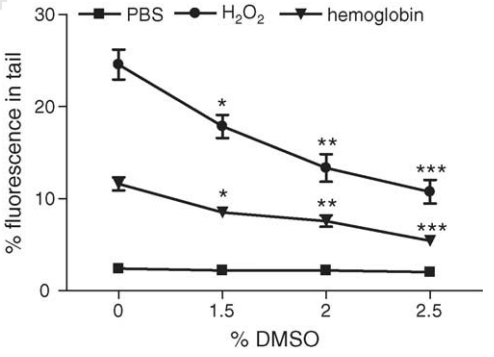

Fig. 1. Significant reduction (one-way ANOVA $p < 0.001$, Bonferroni's multiple comparison test against control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) of DNA damage after adding increasing concentrations of the radical scavenger DMSO to cell suspensions treated with genotoxic concentrations of hemoglobin (1000 μM, 15 min, 37 °C) or H_2O_2 (37.5 μM, 5 min, 4 °C).

did not reach its plateau at the end of the experiment, namely after 24 h, for either of the two compounds. At the same time, total iron concentrations in the medium were reduced. This resulted in the finding that the sum of the curves obtained for cellular and medium-concentrations revealed steady state kinetics for both Fe-donors (data not shown). The absolute iron concentrations (μg per ml or per million cells) were approximately 10-fold different for the two compounds, since 10-fold different concentrations (32 μM for hemin and 250 μM for

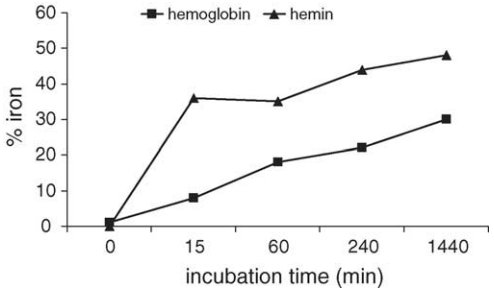

Fig. 2. Rates of cellular iron uptake by HT29 clone 19A cells incubated with 32 μM hemin or 250 μM hemoglobin for 15 min to 24 h. There was a clear time dependant increase of the cellular iron content with a higher relative level after hemin treatment.

hemoglobin) were originally added to the incubation mixtures to account for their different cytotoxic potentials. The comparison of iron uptake by the cells incubated with approximately equitoxic concentrations of both iron sources revealed that more Fe was available from hemin than from hemoglobin (Fig. 2).

4. Discussion

High intake of red meat is most likely associated with increased colon cancer risk, as has first been reported in prospective studies by Willett et al. [20], and then in later reviews of observational and experimental studies and of two meta-analyses [21]. Results of other studies indicated that high consumption of red meat, or of processed meat, in particular, might be associated with an increased risk of cancer of the large intestine [22,23]. Most recently, members of "The European Prospective Investigation into Cancer and Nutrition" (EPIC) study group have provided new information on the intake of meat and colorectal cancer risks [24]. 478,040 men and women from 10 European countries were prospectively followed up who were free of cancer at the time of enrollment between 1992 and 1998. After a mean follow-up of 4.8 years, 1329 incident colorectal cancers were documented and, among others, the relationship was examined between intakes of red and processed meat and colorectal cancer risk. The analysis confirmed the previous studies that colorectal cancer risk is positively associated with high consumption of red and processed meat. The authors reported that the overall association with colorectal cancer risk was, however, stronger for processed meat, than for unprocessed red meat. They discussed this on the basis of heme, which was present in all of the red meat and virtually all of the processed meat studied. Since heme was shown to stimulate production of endogenous *N*-nitroso compounds in the human gastrointestinal system [25] the endogenous *N*-nitrosation, arising from ingestion of heme, was suggested to account for the increased risk of colorectal cancer of the EPIC study.

In the present paper, we have instead hypothesized that hemoglobin and possibly hemin may have other modes of activities in the gut lumen subsequent to red and processed meat intake. Hemoglobin or myoglobin probably release the globin to yield an intact heme complex which then may be absorbed by the intestinal mucosa [26]. Additionally, significant quantities of the dietary heme may be degraded to yield inorganic iron complexes and may be subsequently absorbed in that form [26]. Under in vivo conditions, unabsorbed dietary iron in the gut lumen may be available for Haber–Weiss and Fenton-type reactions to yield radicals from peroxides, which in turn may be genotoxic and pose risks of colorectal cancer [9,10,27]. In accordance with this hypothesis, we have now demonstrated in the present study that hemoglobin is genotoxic.

The genotoxicity of hemoglobin was observed at subtoxic concentrations, whereas, DNA damage, induced by hemin, was detected only with concomitant cytotoxicity. In HT29 clone 19A cells, as well as in primary colon cells, hemoglobin (up to 1000 μM, 15 min of exposure) was non-cytotoxic according to the trypan blue exclusion assay, which measures membrane damage. In contrast, hemin was cytotoxic in the trypan blue exclusion assay at 1000 μM, but only in HT29 clone 19A cells and not in primary colon cells. These findings support conclusions from studies with rats fed purified diets supplemented with hemin, which showed that fecal water from these rats had cytolytic properties [28,29]. The different cytotoxic potential between hemoglobin and hemin was also apparent in another test system, which detected cytotoxicity by measuring the impairment of cell growth (and which could only be performed in HT29 clone 19A cells, since primary cells do not proliferate in culture). Here, only 10 μM hemin, but 50–100 μM hemoglobin, were needed to significantly impair cell growth. The differences between the cytotoxic potentials, and especially the cause of the relatively lower membrane damaging potential of hemoglobin in HT29 clone 19A cells, could be due to the kinetics of a gradual release of toxic hemin from hemoglobin. The lack of membrane damaging cytotoxicity in primary cells may be due to a better cellular uptake of hemin by mechanisms that are more expressed in primary colon cells [26,30], than in tumor cells. When assessing cytotoxicity in both cell types by measuring metabolic activity, the differences between the cytotoxic potentials of the compounds are not as apparent. In this case, already low concentrations

of both compounds (≥100 μM) reduced metabolic activity in HT29 clone 19A cells and in primary colon cells. This may reflect different cellular kinetics for intracellular toxic activities in comparison to extracellular caused membrane damage.

Genotoxic effects were caused by hemin in both cell types at 1000 μM, which was a cytotoxic concentration according to the measurements used in this study. In contrast, hemoglobin was genotoxic also at lower concentrations (≥10 μM in primary colon cells, ≥500 μM in HT29 clone 19A cells). These concentrations were non-cytotoxic in primary human colon cells, which here were used as models for possible mechanisms related to initiation of cell transformation. It is important to relate the employed concentration ranges and the genotoxic/cytotoxic effective concentrations of hemoglobin and hemin to the possible exposures found in the gut lumen. One thousand micromolars was the highest concentration that has been tested. This amount is only threefold more than the levels that had been reported to occur in the gut lumen after iron supplementation. Thus, the genotoxic dose of only 10 μM is well within the physiological concentration range [12,31]. For example, Lund et al. reported that the concentration of water-soluble iron in the gut lumen is normally around 25 μM, but can rise to >100 μM in human feces, with a total concentration of iron in the intraluminal pool reaching 350 μM after oral supplementation of ferrous sulfate [12,31]. Pierre et al. fed rats (treated with azoxymethane) with meat containing low, medium and high heme levels and with ferric citrate and hemoglobin [32]. They found that the heme content in freeze-dried feces and fecal water correlated to the intake and reached levels of 19–1097 μM. In a study by Sesink et al., the feeding of rats with a purified diet supplemented with 1.3 μmol/g of hemin resulted in significantly higher fecal levels of iron (257 μM) than in the controls (80 μM) [28]. Thus in both cases the reported fecal iron concentrations were also within our cytotoxic and genotoxic concentration ranges.

Our studies additionally provide evidence, that hemoglobin also exerts genotoxic effects via the generation of free radicals, since its genotoxicity was markedly impaired in the presence of increasing concentrations of the radical scavenger DMSO. It is possible that reactive oxygen species were formed from peroxides or lipids by the catalytic activity of heme iron [33,34]. Also, radicals arising directly from hemoglobin have been reported to occur [35]. With our studies, it cannot be estimated at which proportion hydroxyl radicals were generated. They should cause pro-oxidative activities with H_2O_2, which was not observed here. The finding however points to a possible mechanism of dietary chemoprotection against iron-mediated effects in the colon, in vivo. On the basis of generally accepted knowledge, it could now be expected that consumption of vegetable and fruits, which are high in antioxidant food ingredients, could counteract the activities of iron in the gut by scavenging free radicals. This has not directly been demonstrated yet. Indirectly, however, it has been shown that the fecal matrix is capable of generating reactive oxygen species in abundance [11]. The free radicals may be dietary-related since another group demonstrated an enhanced formation of free radicals in fecal water of subjects consuming a diet rich in red meat and fat. There was a 13-fold higher production of reactive oxygen species in comparison to fecal water from the same individuals who in a later intervention phase consumed a diet high in dietary plant foods [36]. Using the comet assay, we studied the same fecal waters for their genotoxic activities in human colon cells. We were able to observe a reduced level of DNA damaging agents in the fecal water from the vegetable intervention phase in comparison to the fecal waters derived during the high meat/fat consumption period [37]. More recently we have performed an intervention trial with breads supplemented with prebiotics ± antioxidants and determined different biomarkers of genotoxicity and oxidative DNA damage [38]. The measurements included fecal water genotoxicity, which reflects the exposure situation in the colon lumen. Fecal water genotoxicity was reduced in non-smokers although there was no detectable difference between fecal waters from subjects consuming prebiotic breads and those consuming breads additionally supplemented with antioxidants. Presently, these studies do not answer the specific question on whether dietary antioxidants are capable of reducing damage from hemoglobin-derived radicals in the human gut lumen. They do, however, point to the possibility that this mechanism could take place, although it will be necessary to perform more focused intervention trials in the future to resolve the issue.

According to the hypothesis, cellular absorption of iron is the basis for detecting genotoxic potentials of hemoglobin and hemin in human colon cells. The results presented here indeed do indicate that exposure with hemin and hemoglobin can lead to increased cellular concentrations of iron in HT29 clone 19A cells. After 15 min treatment, already 8 and 36% of the supplemented iron had been absorbed from hemoglobin and from hemin, respectively. Compared to this, the treatment of HT29 clone 19A cells with Fe-NTA (250 μM) resulted in an uptake of almost 50% of the same dose after 15 min [13]. These relative absorption values (Fe-NTA > hemin > hemoglobin) are inversely related to the

molecular weights of the compounds, which may be of importance for cellular bioavailability. Absorption of iron mainly takes place in the duodenum and proximal jejunum, a process, which is well controlled since specific iron-excretion pathways do not seem to exist. In rats fed purified control diets, or purified diets supplemented with 1.3 μmol/g of hemin, protoporphyrin IX, ferric citrate, or bilirubin ($n = 8$/group) for 14 days Sesink et al. have studied iron absorption and effects of iron in the colon lumen. They showed that the apparent iron absorption (occurring in the small intestine) was about 12% of dietary intake in the control group and that the additional uptake of iron from supplemental hemin and ferric citrate was very low [28]. This means that the majority of dietary heme iron and iron from ferric citrate reached the colon. The authors were then able to conclude that heme iron and not inorganic iron was probably responsible for the fecal water cytotoxicity and increased colonic epithelial proliferation observed in that study.

Heme-iron is absorbed by the intestinal mucosa as the intact heme complex [26,30] and Fe (II) is then liberated in the cell via hemoxygenases [39]. Bioavailability of heme in humans was estimated to be up to 35% and at least 65% of total ingested heme iron reaches the colon (reviewed in [26]). In biological systems iron frequently exists as its insoluble ferric Fe (III) form, which may arise by decomposition of heme and which is thus also related to red meat. The cellular uptake of inorganic iron is mediated by transport systems, which require the presence of the ferrous Fe (II) ion, which is very unstable and quickly oxidizes to ferric iron. To enable absorption, specialized transmembrane electron transport systems evolved, known as ferric-chelate reductases. They function by reducing ferric Fe (III) to the ferrous Fe (II) form at the extracellular surface, thus allowing the cell to take up the ferrous iron [40]. This can then be transported into the cell by the transport protein DMT1 (divalent metal transporter 1) [41]. HT29 clone 19A cells express DMT1 [42], which contributes to the efficient cellular uptake we have demonstrated here. Whether or not primary colon cells, as used here, are better equipped to take up the heme complex from hemoglobin, as suggested by our findings, is not known and will need to be resolved in future studies.

In conclusion, the data here presented show that hemin was cytotoxic to human colon cells. There was also a marked genotoxic potential of hemoglobin in primary colon cells at non-cytotoxic concentrations. The studies of mechanisms revealed that hemin and hemoglobin increased iron concentrations in human colon tumor cells and that the observed genotoxicity is probably related to formation of radicals. Thus our studies give new experimental support for the hypothesis that red meat (as a source of hemoglobin, hemin, and iron) contributes to the carcinogenic process through initiation of non-transformed cells and enhanced progression of transformed cells.

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2.2 Publikation II: BUTYRATE MAY ENHANCE TOXICOLOGICAL DEFENCE IN PRIMARY, ADENOMA AND TUMOR HUMAN COLON CELLS BY FAVOURABLY MODULATING EXPRESSION OF GLUTATHIONE S-TRANSFERASES, AN APPROACH IN NUTRIGENOMICS.

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Buttersäure, eine kurzkettige Fettsäure, die während der bakteriellen Fermentation von Ballaststoffen im Dickdarm gebildet wird, vermag das Kolonkrebsrisiko durch Hemmung der Proliferation und Induktion von Apoptose in Tumorzellen zu vermindern. Des Weiteren werden Glutathion-*S*-Transferasen in Tumorzellen durch Butyrat induziert, wodurch die Entgiftung von Karzinogenen gesteigert werden kann. In der vorliegenden Studie wurde die basale Genexpression von 96 Biotransformations-Genen mittels Gen-Arrays in verschiedenen Zelltypen verglichen sowie deren Modulierbarkeit durch eine Butyratbehandlung. Diese Untersuchungen sollten herausstellen, ob Gene für Entgiftungsenzyme auch in primären nicht-transformierten Kolonzellen induzierbar sind.
Die Spiegel einiger GST konnten durch Butyrat positiv beeinflusst werden. Eine Verbesserung der Entgiftungskapazität könnte demnach zu den chemopräventiven Eigenschaften von Butyrat im Kolon beitragen.

Eigenanteil:

- Isolierung und Aufarbeitung der primären Kolonzellen aus Gewebeproben
- Etablierung und Durchführung der Kurzzeitprimärzellkultur sowie Inkubationen dieser Zellen mit Butyrat
- RNA-Isolation, Durchführung der Gen-Arrays und anschließende Genexpressionsanalyse (Datenauswertung, Interpretation und Darstellung)
- Anteilige Verfassung des Manuskriptes

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Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione *S*-transferases genes, an approach in nutrigenomics

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Butyrate, formed by bacterial fermentation of plant foods, has been suggested to reduce colon cancer risks by suppressing the proliferation of tumor cells. In addition, butyrate has been shown to induce glutathione *S*-transferases (GSTs) in tumor cell lines, which may contribute to the detoxification of dietary carcinogens. We hypothesize that butyrate also affects biotransformation in non-transformed colon cells. Thus, we have investigated the gene expression of drug metabolism genes in primary human colon tissue, premalignant LT97 adenoma and HT29 tumor cells cultured in an appropriate medium±butyrate. A total of 96 drug metabolism genes (including 12 GSTs) spotted on cDNA macroarrays (Superarray®; $n = 3$) were hybridized with biotin-labeled cDNA probes. To validate the expression detected with Superarray®, samples of LT97 cells were also analyzed with high density microarrays (Affymetrix® U133A), which include biotransformation genes that overlap with the set of genes represented on the Superarray®. Relative expression levels were compared across colon samples and for each colon sample±butyrate. Compared with fresh tissue, 13 genes were downregulated in primary cells cultivated *ex vivo*, whereas 8 genes were upregulated. Several genes were less expressed in LT97 (40 genes) or in HT29 (41 and 17 genes, grown for 72 and 48 h, respectively) compared with primary colon tissue. Butyrate induced *GSTP1*, *GSTM2*, and *GSTA4* in HT29 as previously confirmed by other methods (northern blot/qPCR). We detected an upregulation of GSTs (*GSTA2*, *GSTT2*) that are known to be involved in the defence against oxidative stress in primary cells upon incubation with butyrate. The changes in expression detected in LT97 by Superarray® and Affymetrix® were similar, confirming the validity of the results. We conclude that low GST expression levels were favourably altered by butyrate. An induction of the toxicological defence system possibly contributes to reported chemopreventive properties of butyrate, a product of dietary fibre fermentation in the gut.

Abbreviations: ARE, antioxidant responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSTs, glutathione *S*-transferases; HBSS, Hank's balanced salt solution; HDACs, histone deacetylases; Keapl, Kelch-like ECH-associated protein 1; PBS, phosphate buffered saline.

Introduction

The lifetime colorectal cancer risk in the general population is reported to be ~5%, with individual risk increasing significantly with age (1). Although a small proportion of colorectal tumors are caused by inherited genetic alterations (2), the greatest numbers of tumors are sporadic and probably the result of a life-long accumulation of genetic alterations in somatic tissues (3,4). These may be caused by carcinogenic compounds derived from foods that are putative risk factors for colorectal cancer (5,6). Carcinogenic compounds ingested with food may pass directly into the gut lumen or may reach the colon indirectly through the bile and/or the enterohepatic circulation after being metabolically activated and conjugated in the liver (7–9). The extent of dietary exposure, the ability to prevent DNA damage by inactivating dietary carcinogens and the capacity to repair the damage caused by dietary carcinogens all contribute to an individual's risk of developing cancer. A favourable balance of biotransformation enzymes, which include various phase I enzymes (10), phase III transport systems (11,12), as well as phase II enzymes, such as glutathione (GSH) *S*-transferases (13), UDP-glucuronosyl transferases (14,15), might protect tumor target cells from accumulating additional mutations. This mechanism of action by xenobiotics has been defined as 'blocking agent activity' as opposed to 'suppressing agent activity', which targets altered cells by e.g. inhibiting their growth or inducing apoptosis (16). Both mechanisms contribute to the chemopreventive action of compounds (17–19).

Levels of biotransformation enzymes have been associated with genetic polymorphisms (20), as well as with environmental factors (21). The induction of selected phase II enzymes that exhibit mainly detoxifying activities is an important target in dietary chemoprevention (22,23). A family of enzymes that plays an important role in detoxification is glutathione *S*-transferases (GSTs; EC 2.5.1.18), which catalyze the conjugation of many electrophilic compounds with reduced GSH. Based on their biochemical, immunological and structural properties, the GSTs are characterized as cytosolic, mitochondrial and microsomal enzymes. The cytosolic transferases are represented by classes Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega. The mitochondrial transferase is called class Kappa GST. The microsomal transferases form a unique MAPEG (membrane-associated proteins in eicosanoid and GSH metabolism) grouping of transferases (13,24).

In this context, we have been exploring the potential of physiologically available butyrate to modulate the expression levels of GSTs (25; T.Kautenburger, G.Beyer-Sehlmeyer, G.Festag, N.Haag, S.Kuechler, A.Kuechler, A.Weise, B.Marian, W.H.M.Peters, T.Liehr, U.Claussen, and B.L.Pool-Zobel, submitted for publication) and to confer resistance to human colon cells towards the exposure to colon cancer risk factors (26,27). Butyrate is a major product of dietary fibre fermentation by the gut microflora and evidence is

accumulating that it may also be formed from other ingredients of plant foods, such as polyphenols (28). In HT29 cells, butyrate was an efficient inducer of GSTs, particularly GSTP1-1, GSTM2-2 and GSTA4-4 (25–29), whereas in colon adenoma cells butyrate reduced the expression of GSTT1-1 protein, probably by destabilizing the GSTT1 mRNA (T.Kautenburger *et al*., submitted for publication). Additional GST genes may contribute to GSH conjugation within colon cells resulting in cellular protection (30).

In this study we (i) investigated whether GSTs and other biotransformation genes were expressed differently in human colon cells and (ii) determined differences in gene expression owing to butyrate. For this purpose we utilized two types of DNA arrays, both novel developments of functional genomics (31) and assessed the expression levels of 12 GSTs in colon epithelial tissue, primary human colon cells (32), premalignant human LT97 adenoma cells (33) and highly transformed HT29 tumor cells (34). All studied stages were considered to be relevant targets to study the dietary-related colon carcinogenesis, and particularly, HT29 cells have been used in many studies as a model for colon cancer cells. We aimed to enhance the knowledge of biotransformation capacities and the transcriptional regulation by butyrate. This type of nutrigenomics approach will help in expanding our understanding of the mechanisms that mediate the effects of chemopreventive diets in reducing the risk of colorectal cancer (35,36).

Materials and methods

Cell lines and culture condition

The human colon adenoma cell line LT97 was a kind gift from Professor Brigitte Marian (Institute for Cancer Research, University of Vienna, Austria) who established it from colon microadenomas of a patient with familial adenomatous polyposis (33). LT97 was maintained in a culture medium (MCDB 302) containing 20% of L15 Leibovitz medium, 2% FCS (fetal calf serum), 0.2 nM triiodo-L-thyronine, 1 μg/ml hydrocortisone (302 basic medium) supplemented with 10 μg/ml insulin, 2 μg/ml transferrin, 5 nM sodium selenite and 30 ng/ml EGF (epidermal growth factor). HT29 cells were isolated from a colon adenocarcinoma of a female Caucasian (34) and originated from an adenoma colon tissue. It was obtained from the American Tissue Culture Collection (ATCC), Rockville, MD, USA. The HT29 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin (26). LT97 and HT29 cells were grown in T25 flasks and cultivated in a humidified incubator (5% CO_2; 95% humidity, at 37°C). Under these conditions, doubling time for LT97 was 72–96 h; and for HT29 cells, 24 h. Passages 29–32 and 29–46, respectively were used for the experiments.

Primary human colon tissue

Cells and RNA were isolated from patients who had given their informed consent after being admitted to hospital for surgery of colorectal tumors, diverticulitis and colon polyps (25). Mean age (±SD) of the six donors of tissue for direct RNA isolation was 58.5 ± 11.1 years; three of the donors were male and three were female. Mean age (±SD) of the three donors from which colon cells were first isolated before incubation and RNA isolation was 65.7 ± 20.2 years; one of the donors was male and two were female. The Ethical Committee of the Friedrich-Schiller-University of Jena approved the study. Non-tumor colon tissue was stored in HBSS (Hank's balanced salt solution; 8.0 g/l NaCl, 0.4 g/l KCl, 0.06 g/l $Na_2HPO_4 \times 2\ H_2O$, 0.06 g/l K_2HPO_4, 1 g/l glucose, 0.35 g/l $NaHCO_3$ and 4.8 g/l HEPES, pH 7.2), transported on ice to the laboratory within 1 h and worked up immediately. The human colon epithelium was separated from the tissue by a perfusion-supported mechanical disaggregation (32). Epithelial stripes were either conserved for RNA isolation or they were further incubated *in vitro* and treated with butyrate (see below).

Treatment with butyrate

Effects of butyrate on the growth properties of HT29 and LT97 cells and on the expression of *GSTP1*, *GSTM2*, *GSTA4* and *GSTT1* have been assessed in detail perviously (25). Based on these studies, each of the cell types was incubated and treated with the maximum butyrate concentration without affecting the viability and growth rates as had been described previously for LT97 and HT29 cells (T.Kautenburger *et al*., submitted for publication; 26), or as had been established during this study for primary colon tissues/cells. Therefore, the cell-specific, subtoxic and optimal conditions varied in terms of time between plating and treatment, duration of treatment and concentration of butyrate. HT29 cells were plated and after allowing attachment for 24 and 48 h, subjected to treatment with 4 mM butyrate or plain medium. LT97 cells were plated and after allowing attachment for 72 h, treated with 1 and 2 mM butyrate or plain medium. Both cell lines were harvested after a further 24 h treatment. Primary human colon tissue pieces were cultured in petri dishes (35 mm) and after allowing to settle for 15 min, subjected to treatment with 10 mM butyrate or plain medium. After 12 h treatment, the cells were isolated from the epithelial stripes by mincing and were incubated in 3 ml HBSS (60 min, 37°C) supplemented with 6 mg proteinase K (Sigma; Steinheim, Germany) and 3 mg collagenase P (Boehringer; Mannheim, Germany). The suspensions of primary human colon cells were diluted with HBSS, centrifuged and resuspended in PBS (phosphate-buffered saline; 8 g/l NaCl, 1.44 g/l Na_2HPO_4, 0.2 g/l KCl and 0.2 g/l KH_2PO_4, pH 7.3). Viability and cell yields were determined with trypan blue.

RNA isolation

Total RNA was isolated from primary human colon cells, LT97 adenoma cells and HT29 tumors cells (up to 6×10^6 cells) using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 30–70 μl RNase free water and stored at −20°C. RNA was also isolated from surgical tissue samples, which had been placed into RNA Later solution (Qiagen, Hilden, Germany) immediately after excision. The integrity of the ribosomal RNA and DNA contamination was checked routinely using formaldehyde denaturing RNA gel electrophoresis (1.2%) before proceeding with the further macro and micro array analysis. Protein or phenol contamination and concentration of the total RNA was assessed by determining the ratio A 260:280 spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany).

Macro and micro array analysis

Superarray.® Hybridization was performed on 112 sites (3 blanks, 3 negative reference spots, 10 household genes, and 96 human genes related to drug metabolism) on cDNA gene macroarrays (GEArray Q Series Human Drug Metabolism Gene Array HS11, SuperArray® Bioscience Corporation; Frederick, MD). Genes were classified into functional categories, representing phase I enzymes (cytochrome p450 family, epoxide hydroxylases), phase II enzymes (acetyltransferases, GST, sulfotransferases, and UDP-glucuronosyl transferases and miscellaneous others) and phase III enzymes (metallothioneins and *p*-glycoproteins). A detailed gene list is available on the company's website (http://www.superarray.com/) and in the accessory file to this manuscript. [The studies were done with c-DNA macroarrays, containing sequences of 96 genes related to drug metabolism, the data for the 12 spotted genes belonging to the family of the glutathione *S*-transferases are presented here in more detail, since confirmatory studies are available. Data for the other genes of drug metabolism are available from the accessory data file (http://www2.uni-jena.de/biologie/ieu/et/Dateien/Butyrate_gene.pdf).] Six arrays were used to determine the interindividual variation levels for RNA isolated from six different donors. Three arrays each were used for RNA isolated from three independently reproduced experiments consisting of medium controls and butyrate-treated samples of primary colon cells, LT97 cells and HT29 cells. HT29 cells were investigated both at 24 h after plating and at 48 h after plating to determine differences owing to culture conditions. Work-up of the array was performed according to the manufacturer's protocol. Single-stranded cDNA was synthesized from total RNA (1–3 μg) *in vitro* by using M-MLV reverse transcriptase (Promega, USA). By applying a single-step ampo linear polymerase reaction (LPR) labelling technique, the cDNA was labelled with dUTP-biotin. The cDNA macroarray was hybridized overnight at 60°C with the biotin-labelled cDNA. The hybridized membrane was subjected to chemiluminesence analysis for quantification of the conjugation signals with streptavidin-linked alkaline phosphatase and CDPstar. The resulting signals were captured by CCD camera equipment (Fujifilm LAS-1000, Diana, USA) and analyzed with AIDA array analysis (Raytest GmbH, Germany) program to comprehensively evaluate the differential gene expression of the various samples. Raw data were normalized between 0 and 100% expression, where the signals of the means of the negative controls (areas without spotted gene sequences or with genes not expressed in human cells) equalled 0 and the means of the signals of the positive controls (household genes) were fixed to equal 100%. Thus, the data shown here represent the mean expression levels relative to negative and positive reference genes. Some genes may reach signals over those of the household genes and thus reach values >100%. Negative values are obtained for genes revealing signals below those of the six negative reference spots. Additionally, to enable other comparisons,

the data were also normalized according to two other criteria (data not shown). One was to set the lowest signal to equal 0% and the other was to set the means of signals of all genes to equal 100% (global normalization). The values of 'fold change', obtained for all three normalization procedures, were used to identify differentially expressed genes and butyrate-regulated genes, respectively. This comparison revealed that the first approach was the most sensitive and (based on all confirmatory data) also the most predictive one.

Affymetrix®. Hybridizations were done on Affymetrix U133A gene expression arrays containing probe sets recognizing >14 000 well-characterized human genes. A detailed list of genes is available on the Affymetrix website (http://www.affymetrix.com). Labelled probes for hybridization were prepared from total RNA obtained as described above from LT97 cells. To remove residual contamination with genomic DNA, total RNA samples were treated with DNAse I at 37°C for 30 min followed by repurification through RNeasy columns (Qiagen, Hilden, Germany). Labelling reactions were done following the suggested protocol for the preparation of fragmented biotinylated complementary RNA (cRNA). In short, with all variable points, 5 μg of total RNA, DNase I treated, was used for cDNA synthesis using the T7-promoter primer (Affymetrix). After a second strand synthesis, biotinylated cRNA was obtained by transcription from the double-stranded cDNA with T7-RNA-polymerase (Enzo). Biotinylated cRNA was fragmented by treatment with Mg^{2+} directly before hybridization. Hybridization and scanning were done on an Affymetrix array processing station and scanner. Primary data obtained scanning the signals of the micro arrays (Affymetrix U133A) were analyzed using the Affymetrix MicroArraySuite analysis package. The resulting signal intensities for each gene and the change of *P*-values were used for comparative evaluation.

Northern blot analysis of GSTP1 expression

Ten micrograms of LT97 and HT29 RNA were loaded on a 1.5% denaturing agarose gel, separated for 3–4 h at 80 V and blotted on a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). Preparation of digoxygenin-labelled RNA probes for GSTP1 and GAPDH was performed as described previously (26). Hybridization occurred overnight at 72°C in standard high SDS hybridization buffer (containing 100 ng/ml of *GAPDH* and 67 ng/ml of *GSTP1* RNA probe). The signals were detected by incubating the membrane with anti-DIG alkaline phosphatase antibody (Roche Diagnostics, Mannheim, Germany), followed by CDP-Star substrate incubation. Afterwards, the blot was exposed for 10 min on X-ray film (Hyperfilm ECL, Amersham Biosciences, Freiburg, Germany) and photographed (Fluor-S® MultiImager, Bio-Rad, München, Germany). Evaluation of the band intensities proceeded with the Quantity One® 4.1 Software (Bio-Rad, München, Germany).

Real-time RT–PCR analysis of GSTT2 expression

Expression of *GSTT2* mRNA was assessed by the two-step SYBR Green I quantitative real-time RT–PCR by iCycler iQ system (Bio-Rad GmbH München, Germany). Briefly, 3 μg of total RNA from the butyrate-treated samples (LT97 and HT29 cells) were converted into first-strand cDNA using Superscript II (Invitrogen) according to the manufacturer's conditions. The PCR amplification reactions contained 2 μl of first-strand cDNA mixed with 12.5 μl of iQ™ SYBR® Green Supermix (Bio-Rad GmbH München, Germany) master mixture (2× mix containing SYBR Green I, iTaq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, 10 mM $MgCl_2$, 20 nM fluorescein and stabilizers), 10 pmol stock of each of the specific primers (*GSTT2*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in a final reaction volume of 25 μl. All reactions were performed in triplicate. The PCR profile consisted of an initial denaturation of 5 min at 95°C, 40 cycles of 30 s at 95°C denaturing, 40 s at 63°C annealing, 45 s at 72°C extension and followed by a final extension step of 10 min at 72°C. Cumulative fluorescence was measured at the end of the extension phase of each cycle. Product-specific amplification was confirmed by melting curve analysis and agarose gel electrophoresis analysis. Gene-specific primer sequences used for the quantification were as follows: *GAPDH*, forward, 5′-CCACCCATGG--CCACCCATGGCAAATTCCATGGC-3′ and reverse, 5′-AGTGGACTCC-ACGACGTACTCAG-3′; *GSTT2* forward, 5′-TGACACTGGCTGATCTC-ATGGCC-3′ and reverse, 5′-GCCTCCTGGCATAGCTCAGCAC-3′; PCR primer for target and reference gene cloning *GSTT2* forward, 5′-GGTGGA-ACGCAACAGGACTGCC-3′ and reverse, 5′-GCCTGATAGGCCTCTGGT-GAGG-3′; and *GAPDH* forward, 5′-CCACCCATGGCAAATTCCATGGC-3′ and reverse, 5′-TAGACGGCAGGTCAGGTCCACC-3′. Primer nucleotides confirmation of the total gene specificity was performed using the BLASTN search programme.

Relative quantification of unknown *GSTT2* mRNA gene expression was determined by using a series dilution of cDNA plasmid containing the inserted *GSTT2*, *GAPDH* and constructing a calibration curve. Wells with no template were used as negative control.

Statistical analysis

Superarray®. Comparisons were made for the directly excised tissue and for colon cells after cultivation in medium, which was a reflection of the baseline expression levels. Comparisons were also made for each of the three colon cells incubated with medium and with butyrate, which was a reflection of the modulated gene expression. Responses of drug metabolism genes spotted on the Superarray® membranes and Affymetrix® array were directly compared by using identical RNA aliquots of LT97 cells incubated in medium and with butyrate. Another comparison was made from a technical point of view, namely to compare the gene expression levels of HT29 cells, which were worked up 48 and 72 h after plating. Genes were clustered into functional entities and subjected to an analysis on a group basis, using the GraphPad® Prism software Version 4.0 (GraphPad® Software Inc., San Diego, USA). Values obtained after normalization were taken for an analyses of variance (ANOVA) test and Bonferroni's post-test was then used to identify genes that were statistically different between the groups. ANOVA calculations taken to compare biopsies and individual cell types were non-repeated measures, whereas ANOVA calculations, based on repeated measures, were used to determine the effects of butyrate. Additionally, unpaired *t*-tests (±Welch's correction for unequal variances) were used, as appropriate, to determine the differences of multiple genes on a group basis. All data were evaluated to establish the two-sided significance levels of independently reproduced determinations.

Affymetrix®. Before comparison of the signal intensities across all data sets, data were normalized using a global normalization approach supervised by the rank intensity distribution of the normalized signal intensities (37). Changes in gene expression were then calculated as fold changes with respect to the untreated reference (38). In cases, in which one gene is represented by different groups of probes, results were summarized when all probe sets gave the same results. In cases of discrepancy, probe set located at the 3′ end of the coding sequence were preferentially considered. In cases where no decision could be made, results for all probe sets were included in the presentation of results. The most likely explanation for these differences is that alternative processed and transcribed mRNA originated from the respective genes.

Real-time PCR. Final results were expressed as an *n*-fold difference in the *GSTT2* gene expression relative to the internal reference *GAPDH* and the calibrator. Statistical significance between control and treated cells was calculated by unpaired *t*-test and one-way ANOVA.

Results

Cellular parameters

Primary cells, isolated from colon tissue incubated *in vitro* (12 h), had a viability of 79 ± 13 and 76 ± 18% in the control medium and in the medium containing 10 mM butyrate, respectively. Confluence of LT97 cells before isolating RNA was ~70–80 and ~80–90% for medium control and for the butyrate-treated samples, respectively. Confluence of HT29 cells after 48 h attachment was 70–80 and 80–90%, and after 24 h attachment it was 60–70 and 70–80%, for medium controls and butyrate-treated samples, respectively. Viability of recovered HT29 and LT97 cells was always >95% for all experimental conditions.

Baseline expression levels

The baseline expression levels of the target genes were determined in freshly excised colon tissues from six individual donors. The data for the GST group of genes obtained for each donor are shown in Figure 1. (The accessory data file shows baseline values for all genes related to drug metabolism.) To enable a better discrimination of the expressed genes, the left panel shows GSTs with low signals and the GSTs with higher relative expression levels are grouped in the right panel of the figure. There was a considerable variation the of expression with total signal strength, which may vary depending on the probe characteristics. Altogether, the signal strength ranged from 482 (donor 3) to 972 (donor 5), which was a 2-fold difference for the sum of all GSTs.

The mean expression levels ($n = 6$ donors) of each individual gene were the basis for assaying differential expression

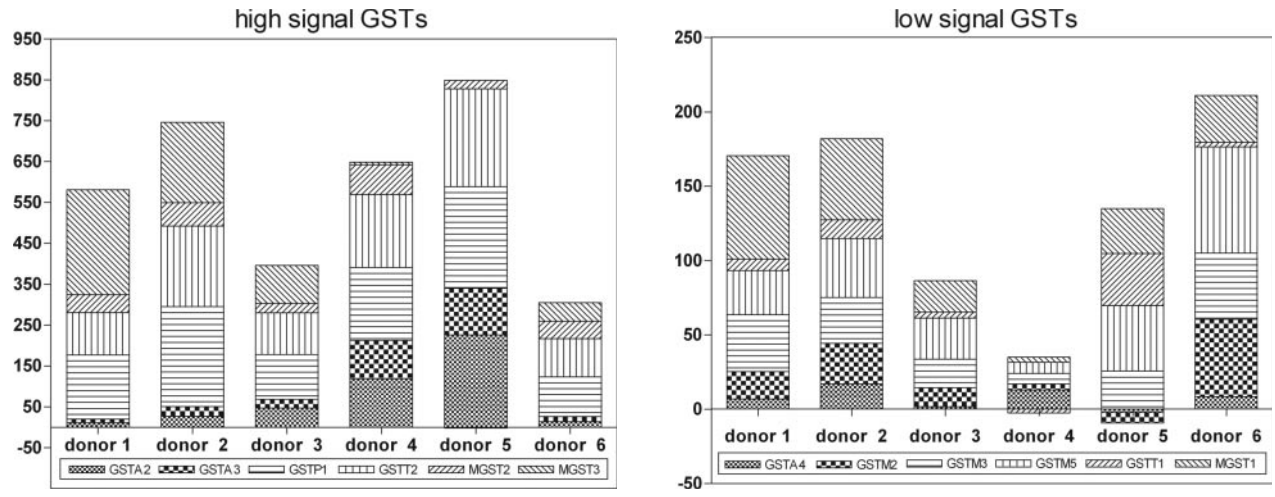

Fig. 1. Individual levels of GST mRNA-expression in freshly excised primary colon tissues derived from six different donors. The 12 GSTs were divided into two groups, namely one with low expression levels and in another one with high expression levels. The mean values and individual variations for the individual GSTs are shown in Table I.

across the human colon samples. For this comparative analysis, we took into account various parameters, namely, the absolute expression levels, the subtractive degree of change between groups, the fold change between groups and the reproducibility of the measurement. The cut-off values for the expression levels were chosen to be 10 [which was equal to the average variation (SEM) in the control genes from the colon tissue samples; $n = 6$]. The fold change to indicate upregulation was set at $\geq$2.0, which was previously found to be significant ($P < 0.05$, $n = 3$) for butyrate-mediated changes of GST activity, *GSTP1* mRNA and GSTP1-1 expression in HT29 cells (25). The fold change to indicate downregulation was set at $\leq$0.5, since this was previously found to be significant ($P < 0.001$, $n = 3$) for the butyrate-mediated inhibition of GSTT1-1 protein expression in LT97 cells (T.Kautenburger, *et al.*, submitted for publication). These numbers were therefore not arbitrary, since they had been shown to reflect significant changes for GST mRNA, GST protein and GST enzyme activity.

Table I shows the relative baseline expression levels for all differentially expressed genes (including GSTs), which were spotted on the membrane (accessory data file). Most genes yielded signals of >10. There were no differences of *GSTP1* and *GSTT1* expression in primary tissues, compared with the colon cells. The other GSTs were differently expressed, albeit with different patterns. With respect to the evaluation criteria described above, a subset of GSTs were less expressed in LT97 and in HT29 (72 h) than in colon tissues. However, in primary cells and in HT29 cells (48 h after plating), there were several examples of genes expressed more in the cells than in biopsies (Table I).

The evaluation of the data on group basis revealed that the expression levels of GSTs from LT97 were significantly ($P = 0.0317$, two-tailed paired *t*-test) different from HT29 (grown for 48 h after plating). Details comparing the expression levels of all genes are available from the accessory data sheets (accessory data file).

Effects of butyrate

According to the exclusion criteria, butyrate was an efficient inducer of GSTs, clearly resulting in an upregulation of *GSTA2* and *GSTT2* in primary cells, of *GSTM3*, *GSTT2* and *MGST3* in LT97 cells and of *GSTP1*, *GSTA4*, *GSTM2*, *GSTM5* and *MGST3* in HT29 cells [Table II (accessory data file)]. With only normalization procedure 1, upregulation of *GSTT2* in LT97 cells (2-fold at 2 mM butyrate) was significant ($P < 0.05$ two-way ANOVA, Bonferroni's post-test), as was the *GSTP1* upregulation (2.2-fold) in HT29 cells incubated for 72 h ($P < 0.001$). On a group basis, 1 and 2 mM butyrate treatment induced GSTs in LT97 ($P = 0.0104$ and $P = 0.0332$, respectively; two-way ANOVA). *MGST1* was efficiently (0.5-fold) downregulated in HT29 cells. It was one of the only few examples of a clear cut downregulation by butyrate, when taking into acount all the genes on the array. Details of all butyrate-mediated effects on GSTs are shown in Table II (accessory data file).

These experiments were independently reproduced three times, thus reducing the necessity of performing the obligatory confirmational experiments usually required for microarray analysis. However, we had some data (e.g. for *GSTP1, GSTM2* and *GSTA4* in HT29) available from previous work, as indicated in the legend of Table II and the expression levels of *GSTP1* in LT97 (no induction in the macro array) and in HT29 (induction in the macro array) were additionally confirmed by northern blot using aliquots of the same RNA. Figure 2 shows that the northern blot results are fully confirmatory of the microarray results. We also subjected an aliquot of the RNA isolated from LT97 (medium control, 1 or 2 mM butyrate) to expression analysis using Affymetrix®. Table III summarizes data for genes which were induced by butyrate using Superarray® ($n = 3$) and which were also spotted on Affymetrix® ($n = 1$). (Table III of the accessory data file shows additional comparisons for genes with signals >10 which were not induced according to Superarray analysis, but which were spotted on the Affymatrix array.) Of these 14 genes, all but one (*GSTT2*) gave results in the same direction. Therefore, *GSTT2* expression was additionally confirmed with real-time RT–PCR.

Analysis of the effect of butyrate on the colon adenoma cell line LT97 with Affymetrix® arrays also shows a significant regulation of a larger number of other genes (~500) connected with various other cellular processes. These data however, do not interfere with the discussed effect on the detoxifying genes and will be presented in another manuscript under preparation.

Table I. Baseline expression levels of drug metabolizing enzymes in human colon cells compared with tissues

Functional gene family	Number of expressed genes	Differentially expressed genes	Primary tissue* 0 h[a]		Primary cells 12 h[a]		LT97 adenoma cells 72 h[a]		HT29 tumour cells 72 h[a]		HT29 tumour cells 48 h[a]	
			Means	SEM	Down	Up	Down	Up	Down	Up	Down	Up
Phase I												
p450 Family	14/25	CYP2B	60.3	21.8	CYP2B		CYP2B		CYP2B		CYP3A4	
		CYP2F1	33.7	7.8			CYP2F1		CYP2F1			
		CYP3A4	36.3	17.5			CYP3A4		CYP3A4			
		CYP4F3	154.5	32.7			CYP4F3					
Phase II												
Acetyltransferases	10/10	ACAT1	89.8	16.1	CRAT	ACAT2	ACAT1		ACAT1		CHAT	DLAT
		ACAT2	331.5	63.3		LOC51126	ACAT2		ACAT2		NAT1	HAT1
		CHAT	134.8	44.2			CHAT		CHAT			HBOA
		CRAT	41.9	12.1			CRAT		CRAT			LOC51126
		DLAT	27.8	7.4			DLAT		DLAT			
		HAT1	17.6	6.6			NAT1		NAT1			
		HBOA	3.2	4.7			LOC51126		LOC51126			
		MORF	17.2	5.1								
		NAT1	82.4	16.6								
		LOC51126	48.2	12.9								
Glutathione *S*-transferases	12/12	GSTA2	73.7	34.4	GSTA2	GSTM2	GSTA2		GSTA2		GSTA2	GSTA4
		GSTA3	45.8	18.8	GSTA3	GSTM3	GSTA3		GSTA3		GSTA3	MGST1
		GSTA4	7.4	2.9	MGST1	GSTM5	GSTM3		GSTM3		GSTM3	
		GSTM2	17.9	8.4			GSTM5		GSTM5		GSTM5	
		GSTM3	27.5	5.5			GSTT2		GSTT2			
		GSTM5	36.7	8.6			MGST2		MGST2			
		GSTT2	152.2	25.1			MGST3		MGST3			
		MGST1	35.1	9.7								
		MGST2	43.7	8.2								
		MGST3	99.3	43.3								
Sulfotransferases	15/21	CHST5	37.8	18.1	CHST5	TPST1	CHST6		CHST6		SULT1B1	CHST5
		CHST7	72.1	45.8	CHST7		HNK-1ST		HNK-1ST			CHST7
		HNK-1ST	56.4	7.9	SULT1B1		SULT1A1		SULT1A1			
		SULT1A1	31.8	11.9			SULT1A2		SULT1A2			
		SULT1A2	27.5	9.5			SULT1B1		SULT1B1			
		SULT1B1	52.4	20.4			TPST1		TPST1			
		TPST1	40.2	4.8								
Miscellaneous	12/13	UGT1A1	151.5	54.0	UGT2A1	NNMT	UGT1A1		UGT1A1	TPMT	UGT2A1	UGT2B
		UGT2A1	28.0	10.0	UGT2B4		UGT2A1		UGT2A1		UGT2B10	TPMT
		UGT2B	59.1	9.7	TPMT		UGT2B		UGT2B		UGT2B4	EPHX1
		UGT2B10	105.5	15.8			UGT2B10		UGT2B10		NNMT	
		UGT2B4	38.8	13.7			UGT2B4		UGT2B4			
		COMT	32.4	8.6			HNMT		COMT			
		HNMT	169.9	20.9			NNMT		HNMT			
		NNMT	87.8	18.3					NNMT			
		TPMT	33.6	25.7								
Phase III												
Metallothioneins	8/8	MT1A	125.5	33.1		MT1G	MT1A		MT1A		MT1A	
		MT1G	257.1	23.2			MT1G		MT1G		MT1G	
		MT1H	196.7	32.6			MT1H		MT1H		MT1H	
		MT1L	267.5	34.1			MT1L		MT1L		MT1L	
		MT2A	87.9	23.6			MT2A		MT2A		MTIX	
		MT3	21.2	4.3			MTIX		MT3			
		MTIX	386.0	42.5					MTIX			
p-Glycoproteins	3/7	ABCC2	127.1	70.4	ABCC2		ABCC2		ABCC2			ABCC3
		ABCC3	45.7	13.0	ABCG2		ABCG2		ABCG2			
		ABCG2	131.0	39.6								
Housekeeping genes	10/10	GAPD	311.2	30.3			PPIA	RPL13A	PPIA	RPL13A		RPL13A
		GAPD	275.3	17.7			PPIA	RPL13A	PPIA	RPL13A		RPL13A
		PPIA	89.5	7.6			PPIA	ACTB	PPIA	ACTB		ACTB
		PPIA	88.4	11.0			PPIA	ACTB		ACTB		ACTB
		PPIA	97.7	6.8								
		PPIA	107.0	4.9								
		RPL13A	−0.3	5.1								
		RPL13A	0.9	4.4								
		ACTB	15.3	7.1								
		ACTB	14.9	7.6								

Primary colon cells isolated from surgical material, LT97 human colon adenoma cells and HT29 cells were plated and grown in medium for 12 h, 72 h, and/or 48 h prior to work-up. Only those genes for which the baseline expression levels reached a signal of $\geq$10 at least in one cell type are show in the table. Regulation is based on expression levels in primary tissue. Downregulation is defined as a ratio $\geq$2 (tissue/cells), and a subtractive difference $>$20. Upregulation is defined as a ratio $\leq$0.5 (tissue/cells). Data were calculated using the relative signals obtained after normalization from samples of six different donors (biopsies) from three independently reproduced experiments (colon cells).

*Means $\pm$ SEM, $n = 6$. The baseline data for this table are in Table I of the accessory data file.

[a]Total culture period.

Table II. Overview on the modulation of expression of drug metabolizing enzymes by butyrate in primary colon cells isolated from surgical material in LT97 human colon adenoma cells and in HT29 cells

Functional gene family	Total number of genes	Primary 12 h[a]	LT97 72 h[a]		HT29 72 h[a]	48 h[a]
		10 mM	1 mM	2 mM	4 mM	
Phase I						
p450 Family	25	**CYP2F1** **CYP3A4** **CYP4F3**** **CYP7A1**	CYP4F3***	**CYP4F3*****	**POR**	POR
Phase II						
Acetyltransferases	10	**ACAT1** CHAT	**ACAT1** CRAT DLAT **NAT1** **LOC51126**	ACAT1 **CRAT** **DLAT** **NAT1** **LOC51126**		**CRAT**
Glutathione *S*-transferases	12	**GSTA2** **GSTT2**	GSTM3 GSTT2 MGST3	**GSTM3** **GSTT2*** **MGST3**	**GSTP1***** *MGST1*	GSTA4 **GSTM2** GSTM5 *MGST1* MGST3
Sulfotransferases	21	HNK-1ST		**CHST5** CHST7 **TPST1**		**TPST1*****
Miscellaneous	13		**UGT1A1*** **TPMT****	UGT1A1 UGT2B **TPMT*****	TPMT**	**COMT**
Phase III						
Metallothioneins	8	MT1E MT1L MT3	**MT1A** MT1E **MT1G** **MT1H** **MT1L** **MT2A** **MTIX**	**MT1A** MT1E **MT1G** **MT1H** **MT1L** **MT2A** **MTIX**	**MT1A** **MT1E** **MT1G** MT1H **MT1L** **MT2A** **MTIX**	MT1A MT1E MT1G MT1L MT3 MTIX
p-Glycoproteins	7	**ABCC2** **ABCC3** **ABCG2**	ABCB1 **ABCG2**	**ABCB1**		

Treatment was for 12 h immediately after explantation (primary cells) or for the last 24 h of the whole cultivation period (LT97 and HT29 cells). Only those genes are shown for which the baseline expression levels reached a signal of $\geq$10. Bold letters: butyrate-mediated change was $\geq$2-fold with subtractive differences $>$20s. Normal letters: butyrate-mediated change was at least 1.5-fold and/or with subtractive differences at least 15. *MGST1* was the only gene which was downregulated with a butyrate-mediated change of 0.5-fold. Data were calculated using the mean relative signals obtained after normalization from three independently reproduced experiments. Individual genes were significantly different from medium controls ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; repeated measures ANOVA with Bonferroni's post-test to compare replicate means by row). The baseline data for this table are in Table II of the accessory data file.
[a]Total culture period.

Confirmatory studies of array GSTT2 gene expression by real-time RT–PCR

The modulation of the *GSTT2* gene was confirmed by an independent measure of mRNA levels. Relative mRNA levels using cDNA macroarray were reasonably consistent with relative mRNA levels determined using real-time RT–PCR, which is more sensitive than northern blot analysis. We found that the relative *GSTT2* expression level was 2.73, 2.52-fold and 2.08-fold in the cells treated with 1 or 2 mM (LT97) and 4 mM (HT29) butyrate, respectively (Figure 3a and b). The increased expression of the *GSTT2* gene was statistically significant (one-way ANOVA and unpaired *t*-test).

Discussion

Colon cell systems

In vitro studies provide important tools to enhance our understanding of hazardous effects by chemicals and to predict the potential consequences of exposure to humans (39). There is also an increasing need to investigate chemicals for mechanisms of beneficial effects on health using *in vitro* methods (40). Colorectal cells and cell lines are highly useful in studying the genotoxic potentials of cancer risk factors (32,41–43), properties of chemoprotective components (27,44–46), as well as their interactions (26,38,47). The majority of such *in vitro* studies have utilized tumor cell lines. Whilst this may be feasible for studies on chemotherapeutic potentials, primary or premalignant cells are needed for studies on chemoprevention. However, it has been hardly possible to study early changes affecting the normal colonic epithelial cells owing to the lack of manageable culture methods for those cells (33). We have recently demonstrated the validity of using intact primary colonic epithelial cells (for 30 min–1 h) as models to assess the genotoxicity of risk factors (32,41,48). We have now extended our methodology to first cultivate the intact tissue *in vitro* and then to isolate cells, which was profoundly successful for retaining cell viability. Thus, we were able to treat primary tissue with butyrate for up to 12 h and then isolate viable cells in sufficient quantity and quality for expression analysis.

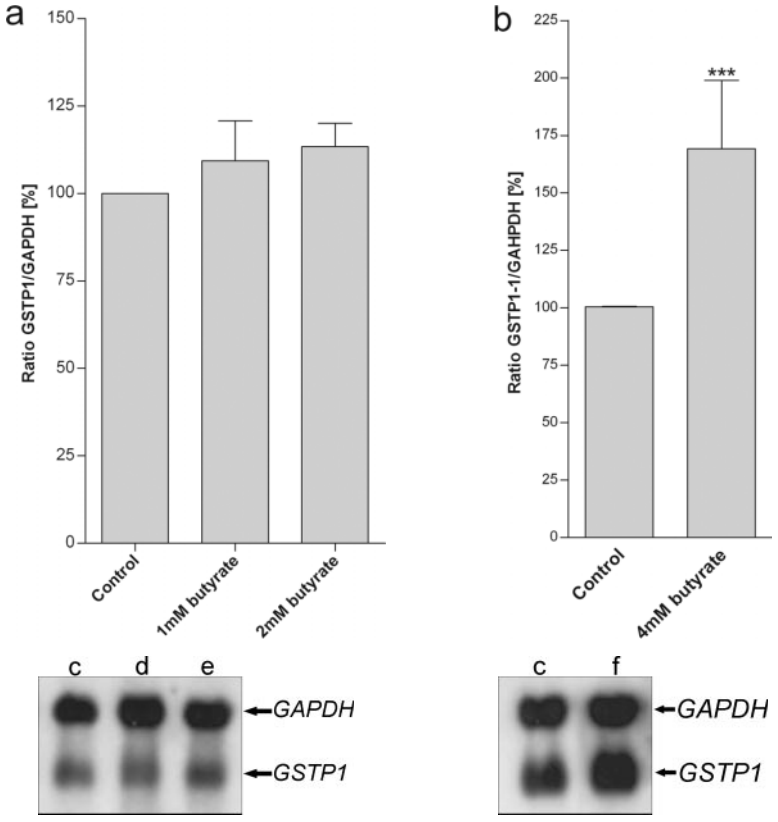

Fig. 2. Northern blots showing the expression of *GSTP1* mRNA in human adenoma LT97 (**a**) and HT29 colon cancer cells (**b**). LT97 and HT29 cells were treated with 1, 2 or 4 mM butyrate. Lane c = control cells, d = 1 mM, e = 2 mM butyrate and lane f = 4 mM butyrate. Values are expressed as mean ±SEM, $n = 3$, $^{***}P < 0.001$ (a: one-way ANOVA, b: unpaired *t*-test).

We had previously also reported novel findings on how a newly established cell line (LT97), consisting of epithelial cells representing an early premalignant phenotype and genotype, could be used as an experimental model to investigate the impact of risk factors (42). The LT97 cells have typical genetic traits of adenoma, such as loss of both *APC* tumor suppressor gene alleles and a mutated *Ki-Ras*-allele, but normal *TP53* (33). This cell line was investigated along with primary and HT29 tumor cells, since there was a need to understand how cell models from these three different stages (non-malignant primary cells, premalignant adenoma cells and malignant tumor cells) would respond to the gut-lumen specific environmental factors.

GSTs in colon tissue

Tissue specimens were included for reference purposes, since this type of material is commonly used to understand gene expression in the human colon and reflects more the expression levels of the *in vivo* situation than cells in culture (49). Here, we have focused our attention on the expression of GSTs (accessory data file), since phase II metabolism is decisive for cell and tissue-specific susceptibility. The activities of both toxic and antitoxic agents are probably highly dependent on the expression of such biotransformation enzymes by which they can be detoxified and which, in turn, they may modulate (50,51). Information on gene expression levels is only available sporadically and data usually pertain to only selected individual genes. We have therefore used expression genomics to enhance our understanding on GSTs [and other drug metabolism systems (accessory data file)] in colon tissue and cells. These advanced methods are very powerful in that they can generate expression data for a large number of genes simultaneously across multiple samples. Here, we have been able to show the GST gene expression levels in tissue directly excised from the colon of six different individuals. The variation on transcriptional level was in a similar order of magnitude as we had previously observed for GST protein expression in colon samples obtained from 15 donors (25). In these previous studies, some samples contained 2- to 4-fold higher GST protein levels than others and in some of the samples, not even the most common colonic GST form, namely GSTP1-1 was available in abundant amounts. The results of this study (mRNA from 12 GST isoenzymes, 6 donors) also show a 2-fold difference between the person with the lowest and highest values of GST expression. On the basis of our present knowledge, we may conclude that a considerable number of subjects could be at higher risk on account of low GST expression levels.

GSTs in cell models compared with tissues

We have now, for the first time, been able to study the expression levels in cells isolated from the tissues (primary cells) incubated *in vitro* for a period up to 12 h. This was the longest duration of *in vitro* culture yielding sufficient viable cells that had succeeded in our hands so far. Several genes were differently expressed in the primary cells, compared with tissues. Three of the 12 investigated GSTs (*GSTA2, GSTA3,* and *MGST1*) were expressed less, whereas three GSTs (*GSTM2, GSTM3* and *GSTM5*) were expressed more in the cells than in the tissue, reflecting changes probably owing to the cultivation

Table III. Modulation of gene expression in LT97 adenoma cells by butyrate

Gene		Superarray Fold change			Affymetrix Fold change		
		1 mM	2 mM		1 mM	2 mM	
Phase I							
p-450 family	CYP3A7	1.7	**2.2**	CYP3A7	0.7	0.7	211843_x_at
	CYP4F3	1.7	**2.4**	CYP4F3	**3.7**	**6.5**	206514_s_at
Phase II							
Acetyltransferases	ACAT1	**4.0**	**3.5**	ACAT1	1.7	1.9	205412_at
	CRAT	**2.4**	**2.6**	CRAT	**2.2**	1.9	209522_s_at, 205843_x_at
	DLAT	**3.9**	**4.9**	DLAT	1.1	1.1	213149_at, 212568_s_at
	HAT1	**2.4**	**2.7**	HAT1	0.6	1.0	203138_at
	LOC51126	**3.4**	**4.4**	LOC51126	1.4	1.5	203025_at
	NAT1	**3.6**	**4.5**	NAT1	**2.1**	**3.6**	214440_at
Glutathione *S*-transferases	GSTA4	**6.1**	**2.7**	GSTA4	**2.2**	**1.7**	202967_at
	GSTM2	**3.2**	**3.5**	GSTM2	**0.6**	**0.7**	204418_x_at
	GSTM3	**5.8**	**7.3**	GSTM3	**3.9**	**4.3**	202554_s_at
	MGST3	**2.0**	**2.7**	MGST3	1.1	1.1	201403_s_at
	GSTT2	1.5	**2.0**	GSTT2	0.9	1.8	205439_at
Sulfotransferases	CHST5	1.4	**2.0**	*CHST5*	1.2	0.8	64900_at
	TPST1	**2.7**	**3.9**	*TPST1*	1.6	1.9	204140_at
	CHST7	1.2	1.6	CHST7	**6.7**	**10.4**	206756_at
Miscellaneous	EPHX1	**2.9**	**2.9**	EPHX1	**2.6**	1.5	202017_at
	LTA4H	**3.2**	**3.7**	LTA4H	1.9	**2.3**	208771_s_at
	UGT1A1	**2.5**	1.8				
	COMT	**2.3**	**2.5**	COMT	**0.3**	**0.4**	208818_s_at, 208817_at
	HNMT	**2.1**	**2.0**	HNMT	**0.4**	**0.2**	204112_s_at, 211732_x_at
	TPMT	1.6	**2.2**	TPMT	1.7	1.4	203671_at, 203672_x_at
Metallothioneins	MT1A	**3.8**	**2.9**				
	MT2A	**6.8**	**4.9**	MT2A	**4.8**	**5.7**	212185_x_at, 212859_x_at
	MT1L	**5.0**	**4.4**				
	MT1G	**2.7**	**2.3**	MT1G	1.9	**4.5**	204745_x_at, 210472_at
	MT1H	**2.6**	**2.2**	MT1H	**4.9**	**6.1**	206461_x_at
	MT3	**2.6**	**4.2**	*MT3*	**2.9**	**4.5**	205970_at
	MTIX	**4.1**	**3.8**	MT1X	**6.3**	**8.8**	204326_x_at, 208581_x_at
Phase III							
p-Glycoproteins	ABCB1	**169.7**	**205.4**	ABCB1	**2.4**	1.5	209994_s_at, 209993_s_at
	ABCC3	0.7	**0.4**	ABCC3	**0.1**	**0.0**	208161_s_at, 209641_s_at
	ABCG2	−19.7	−14.6	ABCG2	**2.2**	**2.0**	209735_at

Comparison of two array methods for those genes which were changed (≥2 fold in comparison to the respective medium control). For the Superarray® membranes, all genes (including those with expression levels <10) were regarded in this evaluation. Bold lettering means that the values are ≥2- or ≤0.5-fold change (butyrate-treated sample/medium control), which is defined as induction or inhibition, respectively. Fold change—mean value of probe sets named in Affymetrix® array (Superarray® $n = 3$; Affymetrix® $n = 1$).

in vitro. In comparison, LT97 and HT29 cells cultivated for 72 h before work-up revealed more striking differences to the tissue samples. Of the 12 GSTs, 7 and 7 respectively, were expressed less in the cells than in the tissue. The differences, however, were not cell line specific, since in HT29 cells cultivated for only 48 h, four genes were expressed less and two additional genes were expressed more. These results again clearly show that the *in vitro* cultivation conditions had marked influence on gene expression, and thus these need to be carefully controlled during experiments using cells *in vitro*.

GST upregulation as a mechanism of chemoprevention

The described comparative analyses (tissue versus colon cells) were needed as a basis to set up the experimental conditions of further studies. These studies had the aim to define specific GST expression patterns in human colon cells of various origins, to determine whether these could be modulated by butyrate, and to assess whether the modulation would be likely to confer protection against diet-associated risk factors. Thus, we have now found that butyrate is an efficient inducer of several GSTs in cells from all three stages of malignancy. Treatments with the maximal tolerated doses of butyrate resulted in an upregulation of *GSTP1, GSTM2*, *GSTA4, MGST3* and others in HT29 cells, of *GSTM2, GSTM3*, *GSTT2* and *GSTA4* in LT97 cells and of *GSTA2* and *GSTT2* in primary cells, with a marked downregulation of *MGST1* in HT29 cells. According to the available databases, the products of these genes inactivate endogenous α, β-unsaturated aldehydes, quinones, epoxides and hydroperoxides formed as secondary metabolites during oxidative stress and protect from food contaminants, such as polycyclic aromatic hydrocarbons (24). For instance the gene product GSTA4-4 was previously reported to have a high affinity for the substrate 4-hydroxynonenal (HNE) (52,53), which is a cytotoxic and mutagenic lipid peroxidation product associated with oxidative stress (54). GSTA2-2 may be of similar importance as GSTA4-4, but with different substrate specificity, resulting in the detoxification of other products, such as cumene hydroperoxide, dibenzo[*a*,*l*]pyrene diol epoxide, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (24). GSTP1-1 may inactivate benzo[*a*]pyrene-9,12-diolepoxide, the reactive intermediate of benzo[*a*]pyrene (55), which is of dietary relevance, since it may be found in cooked foods (56). Other preferential substrates for GSTP1-1 are acrolein, base propenals,

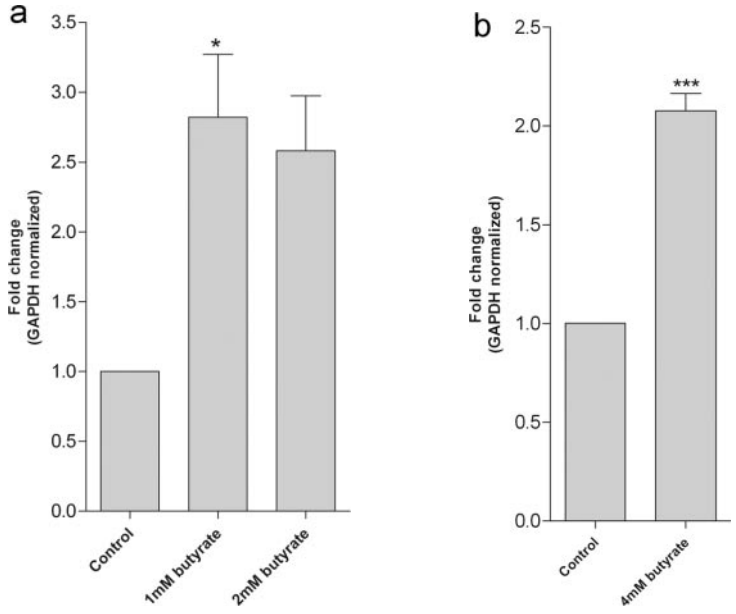

Fig. 3. Quantitative analysis of *GSTT2* mRNA transcript by real-time RT–PCR in LT97 cells (**a**) and HT29 cells (**b**) treated with 1, 2 and 4 mM butyrate, respectively. The relative gene expression analysis in HT29 cells showed 2.08-fold ($^{***}P < 0.001$) and in LT97 cells 2.73 ($^{*}P < 0.05$), 2.52-fold ($^{*}P < 0.05$) (a: one-way ANOVA, b: unpaired *t*-test) increase compared with control cells.

chlorambucil, crotonyloxymethyl-2-cyclohexenone (COMC-6), ethacrinic acid (EA) and thiotepa (24). GSTM2-2 is known to efficiently detoxicate *O*-quinones (e.g. aminochrome), the oxidation products of catecholamines, which may be involved in the development of the Parkinson's disease (57). Other GSTM2-2 substrates are COMC-6, DCNB, dopa *O*-quinone and prostaglandins (24). The human class GSTTs display activity against a broad range of compounds, including methyl halides and sulfate esters. The activity of recombinant GSTT2-2 with a range of secondary lipid peroxidation products, as well as its reported glutathione peroxidase activity with organic hydroperoxides, suggests that it may play a significant role in protection against the products of lipid peroxidation (58), and its substrates cumene hydroperoxide and menaphthyl sulfate (24). Finally, the *MGST3* gene encodes an enzyme, which catalyzes the conjugation of leukotriene A4 and reduced GSH to produce leukotriene C4. This enzyme also demonstrates GSH-dependent peroxidase activity towards lipid hydroperoxides (59) and conjugates CDNB and (*S*)-5-hydroperoxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid (24). *MGST1* encodes a protein that catalyzes the conjugation of GSH to electrophiles and the reduction of lipid hydroperoxides (60). Particular substrates are CDNB, cumene hydroperoxide, hexachlorobuta-1,3-diene (24). *MGST1* was inhibited in HT29 cells, and thus the only example of a clear cut downregulation by butyrate when regarding all genes on the array. The consequences of the downregulation of this GST are not predictable and need to be studied in more detail.

Altogether, a number of the butyrate target genes can be associated with potential chemoprotection, since they should have the ability to ward off risk factors associated with oxidative stress and genotoxic risks (61). Their life-long upregulation in primary cells by dietary butyrate may therefore contribute to the prevention of carcinogenesis, which may be mediated by genotoxic products of oxidative stress (62,63). The ingestion of dietary fibres providing sufficient luminal butyrate concentrations may accordingly be considered to substantially contribute to an effective strategy of dietary cancer chemoprevention.

Confirmatory data

Altogether the evaluation of our expression analysis data was based on $n = 3$ independent experiments (cell culture experiments) or on the data obtained by $n = 6$ individual donors, strengthening the validity of the measurement. We compared different normalization procedures and stick to the one basing the 100% value on the means of all housekeeping genes. The reported responses found with GSTs using this normalization have largely been confirmed independently with other methods. For example, we have previously found that GSTP1-1, GSTM2-2, GSTA1/2 proteins and *GSTM2* mRNA were induced in HT29 cells treated with 2–4 mM butyrate (25), as were *GSTA4* mRNA and GSTA4-4 protein (29). In LT97 cells, GSTP1-1 protein was not induced by butyrate pretreatment (T.Kautenburger *et al*., submitted for publication), the genes found to be modulated in this study (*GSTM3, GSTT2, MGST3*) had not been investigated since antibodies were not available. Other confirmatory data were generated here, such as *GSTP1* induction in HT29 and non-induction in LT97 using northern blot analysis.

Interestingly, the independent evaluation of RNA aliquots using two different array methods gave often similar results, which is to our knowledge, the first direct comparison of this type. An exception was *GSTT2* which was induced according to Superarray®, but not to Affymetrix®. We therefore additionally investigated *GSTT2* expression using another aliquot of the RNA by real-time qRT–PCR. The results confirmed the responses observed for *GSTT2* with Superarray® but not with Affymetrix®. The inability to detect the induction of *GSTT2* on Affymetrix® array is most possibly owing to the characteristics of the *GSTT2* probe set. The signal given by *GSTT2* is so weak that it is assumed by the analysis software as absent both in control and treated cells. However, the signal intensity increased 1.8 times in cells treated with 2 mM butyrate versus control cells.

Potential mechanisms of GST induction in human cells

One important mechanism which is critical for regulation of some, but not all phase II genes (including some GSTs or NADPH-dependent quinone reductase) involves the antioxidant/electrophile-responsive element (ARE/ERE) located within the 5′ upstream (consensus sequences 5′-GTGACNNNGC-3′) regulatory region of the corresponding mouse, rat and human genes (64,65). A major transcription factor which can act on ARE is Nrf2 (nuclear factor E2-related factor 2) (66,67). As a key regulator of Nrf2 activity, which links Nrf2-mediated ARE activation to cellular exposure to oxidants and chemoprotective agents, the BTB- and Kelch-domain containing protein Keap1 (Kelch-like ECH-associated protein 1) has been identified. Keap1 anchors the transcription factor Nrf2 in the cytoplasm and targets it for ubiquitination and proteasome degradation, thereby repressing its ability to induce phase II genes (68). Inducers of ARE-mediated gene expression disrupt the Keap1–Nrf2 complex, leading to an increase in Nrf2 levels, and allowing Nrf2 to translocate into the nucleus (69). Some of the GST-encoding genes contain ARE motif and can be induced in an ARE-mediated manner (24). ARE sequences in the promoter of *GSTA2* are required for basal expression and for its induction by phenolic antioxidants (70). These compounds activate GST-encoding genes, however, also through the AP-1 family of transcription factors, which include Jun, Fos, Maf, ATF and Fra proteins (71). AP-1-binding sites have been identified in the promotor regions of the *GSTA1*, *GSTA4* and *GSTP1* genes (72,73). In addition, C/EBPβ (CCAAT/enhancer binding protein β), which is a member of the C/EBP bZip class of transcription factors, may serve as a more common transcriptional factor for the induction of phase II enzymes and cancer chemoprevention.

The mechanisms by which butyrate probably mediates gene expression in human colon tumor cells are by activation of the mitogen-activated protein kinase (MAPK) signalling transduction pathway (26), and by modifying the acetylation of histones at the N-terminal lysine rich tails (74,75). Two classes of enzymes can affect the acetylation of histones, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs). The classes of compounds that are identified as HDAC inhibitors now include: short–chain fatty acids, such as butyrate and several analogues (76). HDAC inhibitors from several different structural classes exhibit clinical activity against a variety of human malignancies, and have also attracted interest as potential chemopreventive agents. Butyrate inhibits HDAC activity and cell growth at millimolar concentrations. Our own studies have shown that treatment of human colon cells HT29 leads to the marked accumulation of acetylated histone 4 (H4), which could be related to enhanced levels of GST-encoding gene expression in these cells (77). More elaborate studies by Mariadason *et al*. (78) conclude a tight correspondence between the kinetics of altered histone acetylation and kinetics of altered expression for genes in specific clusters and that changes in HDAC activity underlie the changes in expression for these genes. Whether or not promoter areas of GSTs are actually targeted by this mechanism is not known and will be an important subject of research in the near future.

Double-edged sword/GST induction in tumor cells

It remains to be elucidated, whether the typical expression patterns can afford chemoresistance of the cells to appropriate substrates, some of which may be colon cancer risk factors. It must also be clarified for each of the target genes in more detail by which mechanisms their butyrate-mediated induction proceeds on molecular level, e.g. by inhibiting the deacetylation of histones (79) and/or through MAPK pathways leading to the transcriptional activation of antioxidative response element (19).

In this context, we must also again consider the concept of a double-edged sword. On one hand, an induction of GSTs in primary cells seems straightforward and favourable since this should result in an enhanced detoxification of risk factors. Connected to this is a reduced probability of cancer initiation in the underlying stem cells. In tumor and in adenoma cells, on the other hand, GST induction could counteract cancer chemotherapy by causing resistance to therapeutic agents, thereby enhancing the survival of transformed cells (26). However, this adverse situation, may not be probable *in vivo*, since the luminal millimolar concentrations of butyrate could be much too high (exceeding 2–4 mM) to result in GST induction. Instead physiological gut luminal butyrate concentrations would impair tumor cell or adenoma cell growth and thus decrease availability of such cells for GST induction. The physiologically available butyrate amounts may also be efficient in inducing apoptosis in tumor cells and thus additionally remove them from the tissue. Another reflection is that, not only butyrate, but also propionate is produced during gut fermentation, and this short chain fatty acid adds on to the growth inhibitory properties of gut luminal products (80,81). Finally, according to all available information, the concentrations found in the gut lumen are much higher (10–20 mM) than the concentrations used here (82,83), albeit *in vivo* colon tissue is probably more protected from the gut luminal components by barrier functions of the mucosa (84) than they are *in vitro* in cell culture. However, it may still be speculated that emerging premalignant and malignant cells will be removed owing to the toxic and growth inhibitory properties of SCFA before GST induction can occur.

Physiological butyrate concentrations may indeed retard tumor progression and lead to a reduced tumor incidence, as has been suggested by the results of a number of animal studies. Dietary fibres, which are fermented to yield high amounts of butyrate, have been associated with a higher efficacy of protecting from AOM-induced colon tumors in animals (85–88). In particular, an *in vivo* study by Perrin demonstrated that those fibres, which promoted a stable butyrate-producing colonic ecosystem decreased the rate of aberrant crypt foci in rats, thus adding on to the line of evidence that a stable butyrate producing colonic ecosystem related to dietary plant foods reduces risks of developing colon cancer (87).

Conclusions

A considerable number of subjects could be at higher risk on account of low GST expression levels in their colonocytes. The hypothesis is that butyrate may mediate in colonocytes an enhanced expression of GSTs and other systems, which protect from products of oxidative stress. We have now been able to add evidence to support this possible mechanism using new systems of *in vitro* toxicology, namely, primary human colon cells. The favourable modulation of toxicological defence systems in these cells is expected to contribute to protection during early stages of carcinogenesis by resulting

in an enhanced cellular protection from cancer risk factors. Butyrate also has the potential to inhibit growth of emerging premalignant and malignant cells, which could conceivably retard tumor progression. When translated to the *in vivo* situation, it must be first of all be taken into account that results on gene expression regulation in some conditions *in vitro* may misrepresent the status of regulation of the same genes *in vivo*. However, the results are also promising in that they could also mean that a life-long supply with butyrogenic dietary plant foods may contribute substantially to dietary colon cancer chemoprevention. This is a feasible hypothesis, which will be needed to be proved in human clinical trials.

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2.3 Publikation III: PRODUCTS FORMED DURING FERMENTATION OF THE PREBIOTIC INULIN WITH HUMAN GUT FLORA ENHANCE EXPRESSION OF BIOTRANSFORMATION GENES IN PRIMARY COLON CELLS.

Julia Sauer, Konrad K. Richter, Beatrice L. Pool-Zobel. *British Journal of Nutrition.* Angenommen am 30.11.2006.

Fruktooligosaccharide werden von der Darmflora zu kurzkettigen Fettsäuren fermentiert. Ein Hauptvertreter ist Buttersäure, die zahlreiche chemoprotektive Eigenschaften aufweist. Die Wirkung komplexer Fermentationsprodukte wurde bislang noch nicht ausführlich untersucht. In der vorliegenden Arbeit wurde eine *in vitro* Fermentation mit einem Inulin-Oligofruktose-Gemisch als Substrat durchgeführt. Die so hergestellten Fermentationsüberstände wurden auf ihre Konzentration an kurzkettigen Fettsäuren und ihre Wirkungen (zytotoxische oder trophische Effekte, Modulation der Genexpression) auf primäre Kolonzellen getestet. Die Fermentation des Inulin-Oligofruktose-Gemischs führte zu einer deutlichen Erhöhung der Konzentration an kurzkettigen Fettsäuren. Hohe Konzentrationen des Fermentationsüberstandes wurden gut von den primären Kolonzellen toleriert, was auf trophische Effekte hindeutet. Die ermittelte Induktion von GST durch den Fermentationsüberstand könnte nicht-transformierte Zellen vor Kolonkarzinogenen schützen.

Eigenanteil:

- Isolierung und Aufarbeitung der Kolongewebeproben
- Durchführung der Inkubationen mit dem Fermentationsüberstand
- Messung der metabolischen Aktivität
- RNA-Isolation und Durchführung der Gen-Arrays sowie Durchführung der real-time PCR und Messung der GST-Aktivität
- Auswertung, Interpretation und Darstellung der Ergebnisse
- Verfassung des Manuskriptes

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Products formed during fermentation of the prebiotic inulin with human gut flora enhance expression of biotransformation genes in human primary colon cells

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Inulin-type fructans are fermented by gut bacteria to yield SCFA, including butyrate which is trophic for colonocytes and induces glutathione *S*-transferases (GST) that detoxify carcinogens. Since little is known on similar effects by complex fermentation samples, we studied related products in non-transformed human colonocytes. Inulin enriched with oligofructose (1 : 1, Synergy1) was fermented with human gut flora. SCFA were quantified and a SCFA mixture was prepared accordingly. Colonocytes were incubated (4–12 h) with the Synergy1 fermentation supernatant (SFS), faeces control, a mixture of the three major SCFA (each 0–15 %, v/v) or butyrate (0–50 mM). Metabolic activity was determined to assess trophic effects and cytotoxicity. Expression of ninety-six genes related to biotransformation was studied using cDNA macroarrays. Results on modulated GST were reassessed with real-time PCR and GST activity was measured. Fermentation of inulin resulted in 2–3-fold increases of SCFA. The samples were non-cytotoxic. SFS increased metabolic activity, pointing to trophic effects. The samples modulated gene expression with different response patterns. Key results were that *GSTM2* (2·0-fold) and *GSTM5* (2·2-fold) were enhanced by SFS, whereas the SCFA mixture reduced expression. The faeces control enhanced *GSTA4* (2·0-fold), but reduced *GSTM2* (0·2-fold) and *GSTM5* (0·2-fold). Real-time qPCR confirmed the induction of *GSTM2* and *GSTM5* by SFS and of *GSTA4* and *GSTT2* by butyrate. Activity of GST was not modulated. High concentrations of fermentation products were well tolerated by primary colonocytes, pointing to trophic effects. The induction of GST by the SFS may protect the cells from carcinogenic compounds.

Butyrate: Fermentation supernatants: Human colon cells: Glutathione *S*-transferases

Inulin is a mixture of fructans consisting of monomers linearly linked by means of β(2-1) bonds with different degrees of polymerization. This prebiotic cannot be hydrolysed by digestive enzymes in the upper intestinal tract of man (Schneemann, 1999). Therefore, the non-digestible carbohydrates reach the colon where they are fermented by *Bifidobacterium* spp. and other lactic acid-producing bacteria. This results in enhanced concentrations of these bacteria in the gut which beneficially affect the hosts (Ellgard *et al.* 1997; Klinder *et al.* 2004*a*). In particular, a number of different experimental studies have shown that the fermentation products of inulin contribute to colon cancer-preventing properties, as has been summarized in a recent review (Pool-Zobel, 2005). For example, *in vitro* it beneficially modulated markers of tumour progression in a human colon tumour cell model (Klinder *et al.* 2004*b*). *In vivo*, several studies have shown that the addition of inulin to the diet of azoxymethane-treated rats reduced the yield of aberrant crypt foci (Pool-Zobel, 2005). The effects of inulin were dose related, and also dependent on the chain length of the inulins (Verghese *et al.* 2002, 2003).

The consumption of dietary fibre may be indirectly protective in the colon by elevating faecal volume and enhancing defecation, both of which reduce exposure of colonocytes to cancer risk factors. In addition, protection may be a result of the gut flora-mediated fermentation of dietary fibre. The fermentation products of inulin may be protective in early stages of cancer onset since the number of apoptotic cells per crypt was higher in rats fed oligofructose and long-chain inulin (Hughes & Rowland, 2001). Poulsen *et al.* (2002) also reported a decreased cell proliferation and therefore a reduced cell turnover which may be indicative of cancer suppression also at later stages of the progression process.

Some types of dietary fibre including inulin, however, could be protective via the formation of SCFA during fermentation by the gut flora (Cummings, 1981). In non-transformed cells, the SCFA, butyrate, is utilized as an energy source (Roediger, 1989), and in tumour cells, butyrate reduces survival by inducing their apoptosis and inhibiting proliferation (Kruh, 1982). Another mechanism of protection by fermentation products, especially by butyrate, has been

Q1 **Abbreviations:** GST, glutathione *S*-transferase; qPCR, quantitative PCR; SFS, Synergy1 fermentation supernatant.

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hypothesized to be the induction of glutathione *S*-transferases (GST) which are phase II enzymes of biotransformation that detoxify many carcinogens (Turesky *et al.* 1991; Manus *et al.* 1997). The increased cellular levels of such enzyme systems has been shown to protect against food-derived genotoxic compounds such as 4-hydroxynonenal in tumour-derived cell lines (Ebert *et al.* 2001). Similar mechanisms occurring in non-transformed cells may very well reduce cancer initiation, and thus be considered an effective means of primary cancer chemoprevention (Johnson *et al.* 1994; Pool-Zobel *et al.* 2005*b*) since GST are capable of detoxifying endogenous and food-derived carcinogens like 4-hydroxynonenal or benzo(*a*)pyrene.

It is now of interest to explore whether complex fermentation samples (containing butyrate, other SCFA and additional compounds) may have similar activities. Therefore, we investigated fermentation samples from inulin using human non-transformed primary colon cells representing the more appropriate target cells of chemoprevention. We compared the effects to those of butyrate and to a mixture of SCFA composed according to the fermentation sample. The fermentation products studied here were generated *in vitro* using anaerobic procedures that simulate the physiological conditions of the human gut. This presents an experimental approach to analyse the biological activities of different dietary fibres and their resulting complex fermentation products (Wang & Gibson, 1993). The results were expected to enhance our understanding of the chemopreventive properties of prebiotics' fermentation supernatants from diet in terms of reducing colorectal cancer risks (Lupton, 2004).

Materials and methods

Fermentation of inulin-derived prebiotics

The carbohydrate source used in the fermentation experiments was the fructan Synergy1, a commercially available 1 : 1 mixture of inulin (Femia *et al.* 2002) enriched with oligofructose (ORAFTI, Tienen, Belgium). The fermentation of this inulin-type fructan mixture was conducted *in vitro* under anaerobic conditions (80 % N_2, 10 % CO_2, 10 % H_2 at 37°C) in a batch-culture system with faecal inoculum of different donors (Manderson *et al.* 2005; Van Loo *et al.* 2005). Each tube was mixed well and incubated at 37°C for 24 h during which it was shaken manually at intervals. After placing the suspensions on ice to stop the fermentation, the samples were centrifuged at 6000 ***g*** at 4°C for 30 min. The fermentation supernatants were stored at −20°C and filter-sterilized (pore size 0·22 μm) before use in the experiments.

Determination of SCFA

The content of the SCFA of the fermentation supernatant and the corresponding faeces control was determined using GC (Kiessling *et al.* 2002).

In brief, the sample was weighed and an internal standard solution (2-methyl valeric acid, 0·01 %) was added. The solution was acidified with 0·5 ml H_2SO_4 ($pH < 2$) and extracted by shaking with 2·0 ml diethyl ether and subsequent centrifugation (10 min at 2000 rpm). The ether phase was injected directly on to the oven-heated (150°C) column (BT21-FFAPP, Q2 25 m × 0·53 mm × 0·5 mm; Achrom NV/SA, Belgium), the carrier gas used was N_2 and detection temperature was set at 230°C (GC VEGA 6000; Carlo Elba, Milan, Italy). Peaks were Q3 integrated automatically using Atlas software (ThermoLab Systems, The Netherlands).

Preparation of synthetic SCFA mixtures

The analytical data of the SCFA determination were taken as a basis to compose the synthetic Synergy1 fermentation supernatant (SFS) mixture. This was prepared to mimic the amounts and proportions of SCFA found in the inulin-type fructan fermentation supernatant. The concomitant investigation of the synthetic mixture of the major SCFA acetate, propionate and butyrate was expected to reveal which proportion of biological activity was due to the SCFA in the fermentation supernatant.

For this we dissolved sodium butyrate, sodium propionate and sodium acetate in the determined molar concentrations in cell culture medium and prepared 50-fold concentrated stock solutions. Aliquots were stored at −20°C and diluted to the end concentrations applied in the experiments before use.

Primary colon tissue preparation and isolation of cells

Primary colon cells were isolated from colon tissue obtained during surgery of colorectal tumours, diverticulitis and colon polyps from patients who had given their informed consent. The tissue specimens were parts of the non-tumorous tissue which was co-removed for medical indications. The university ethics committee approved the study. Mean age of the three donors of colon cells for incubation and RNA isolation was 66 (SD 20) years. One of the donors was male, two were female. The mean age of the six donors whose cells were used for determining metabolic activity was 57 (SD 19) years. Four of the donors were male, two were female. The cells used for incubation and cytosol preparation (GST activity) were taken from two male and one female subject (mean age 69 (SD 12) years). The tissue was prepared as described previously (Schäferhenrich *et al.* 2003). These epithelial stripes were used for incubation and subsequent analysis of gene expression using conditions that had been determined as optimal before. After 12 h treatment, the cells were isolated from the epithelial stripes as described before (Schäferhenrich *et al.* 2003). Viability and cell yields were determined with trypan blue. We also digested the tissue stripes directly to yield a single cell suspension which was seeded and incubated for the metabolic activity assay.

Measurement of the metabolic activity

Single cells were seeded into ninety-six-well microtitre plates (50 000 cells/well) and were incubated in minimal essential medium enriched with 20 % FCS, 2 mM-glutamine, 1 % penicillin/streptomycin, 100 μg/ml gentamycin, 2·5 μg/ml fungizone, Q4 10 ng/ml endothelial growth factor, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Rogler *et al.* 1998).

Primary colon cells were incubated for 4, 12 and 24 h with SFS (0–15 %) and butyrate (0–50 mM, positive control) to determine the biologically effective concentration ranges. A faeces blank supernatant (fermentation without substrate) was included in each experiment as the negative control.

A mixture of the SCFA (acetate, propionate and butyrate) was diluted in culture medium at concentrations which mimicked the content of SCFA in the SFS. This mixture was also included as a positive control. To measure the metabolic capacity of the cells, they were incubated in ninety-six-well microtitre plates with the dye resazurin which is reduced into resorufin only by viable cells (CellTiter-Blue® assay; Promega). The product is highly fluorescent and was detected with Ex/Em 520/595 nm after 2 h incubation with the reagent. Mean values and standard deviations were calculated of at least three independent experiments.

Gene expression studies

Using concentrations determined to be subtoxic, human colon epithelium stripes were incubated with SFS (10 %), a synthetic mixture of SCFA, a faeces supernatant control, medium or butyrate. The chosen butyrate concentration (10 mM) mimics possible physiological concentrations in the gut lumen (Hass *et al.* 1997). We incubated small tissue pieces in Petri dishes (35 mm) to ensure the greatest possible surface for contact with the medium and to maintain the highest possible viability. After allowing the epithelial stripes to settle for 15 min, they were subjected to treatment with the test compounds.

Total RNA was isolated from the cells (up to 6×10^6 cells) with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at −20°C. The ratio A_{260}/A_{280} and concentration of total RNA were determined spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany) for protein or phenol contamination followed by formaldehyde denaturing RNA gel electrophoresis (1·5 %) to check the integrity of the ribosomal RNA and possible DNA contamination.

Expression analysis of 112 human genes (sixteen reference spots, and ninety-six genes related to drug metabolism) was performed with cDNA gene macroarrays (GEArray Q Series Human Drug Metabolism Gene Array HS11; SuperArray® Bioscience Corporation, Frederick, MD, USA). Genes were classified into functional categories, representing phase I enzymes (cytochrome p450 family, epoxide hydrolases), phase II enzymes (acetyltransferases, GST, sulphotransferases and miscellaneous others which included UDP-glucuronosyl transferases), and metallothioneins and *p*-glycoproteins. A detailed gene list is available at the company's website (www.superarray.com). Three arrays each were used for RNA isolated from the medium controls and the treated samples of primary colon cells. The array was performed according to the manufacturer's protocol and as described previously (Pool-Zobel *et al.* 2005*a*). Raw data were normalized between 0 and 100 % expression whereas the signals of the negative controls were calculated as 0 % and the means of the signals of the positive controls were set to equal 100 %. Thus, the data shown here represent mean expression levels relative to negative and positive reference genes as described previously (Pool-Zobel *et al.* 2005*a*).

Relative quantification of glutathione S-transferase expression with real-time PCR

The expression of several GST isoforms (*GSTA4*, *GSTM2*, *GSTM5*, *GSTP1*, *GSTT2*) found to be modulated according to the array analysis was also studied using quantitative real-time PCR (SYBR Green I system). Total RNA (1 μg) was reverse transcribed (SuperScript II, First-Strand cDNA Synthesis System; Invitrogen) in 20 μl buffer with oligo-$(dT)_{15}$ primers according to the manufacturers' instructions. cDNA (5 μl; 33·3 ng total RNA equivalent) was used in a 25 μl PCR amplification reaction containing 2 × iQ SYBR Green supermix® (100 mM-KCl, 40 mM-Tris-HCl, pH 8·4, 0·4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM-$MgCl_2$, SYBR Green I, 20 nM-fluorescein, stabilizers) and 10 pmol gene-specific primers for the target GST genes and the reference (*GAPDH*) gene. The following primer sequences were used to amplify a region of *GSTA4*, *GSTM2*, *GSTM5*, *GSTP1*, *GSTT2* and *GAPDH* mRNA:

iGSTA4_F 5′-ccg gat gga gtc cgt gag atg g-3′
iGSTA4_R 5′-cca tgg gca ctt gtt gga aca gc-3′
iGSTM2_F 5′-agc cgt atg cag ctg gcc aaa c-3′
iGSTM2_R 5′-cca caa agg tga tct tgt ccc ca-3′
iGSTM5_F 5′-ttg cag gag aca aga tca cct ttg-3′
iGSTM5_R 5′-gat ctt ctt caa acc ctc aaa gcg-3′
iGSTP1_F 5′-ctg cgc atg ctg ctg gca gat c-3′
iGSTP1_R 5′-ttg gac tgg tac agg gtg agg tc-3′
iGSTT2_F 5′-tga cac tgg ctg atc tca tgg cc-3′
iGSTT2_R 5′-gcc tcc tgg cat agc tca gca c-3′
iGAPDH_F 5′-cca ccc atg gca aat tcc atg gc-3′
iGAPDH_R 5′-agt gga ctc cac gac gta ctc ag-3′

PCR cycles included one cycle of 95°C for 2 min followed by forty cycles each of 94°C for 30 s, annealing temperature 60°C for 30 s and 72°C for 40 s, and a final extension step of 72°C for 10 min (iCycler iQ® Real-Time PCR Detection System; Biorad GmbH, Munich, Germany). Product-specific amplification was confirmed by melting curve analysis and agarose gel electrophoresis. All experiments were performed in duplicate. The fluorescence threshold value (C_T) was calculated using the iCycler iQ® optical v3·0a system software. The relative quantification of *GST-mRNA* expression was calculated with the comparative $\Delta\Delta C_T$ ($\Delta\Delta C_T = \Delta C_{Tcontrol} - \Delta C_{Texperiment}$) method. For normalization, ΔC_T values were calculated by subtracting the average of the C_T value in the control for the reference gene from the average of the C_T value for the target gene and subtracting the average of the C_T value in the treated sample of the reference gene from the target gene. Then the difference between the ΔC_T values of control and treatment ($\Delta\Delta C_T$) was calculated. The fold change was calculated according to the efficiency method ($\Delta\Delta C_T$ method) where it is assumed that the PCR efficiency is 100 % ($E = 2$; fold change $= E^{\text{difference}}$; Pfaffl, 2001; Pfaffl *et al.* 2002).

Preparation of cytosol, measurement of total glutathione S-transferase activity and determination of cytosolic protein

Epithelial stripes were incubated for 12 h with 10 % SFS, 10 mM-butyrate and controls, and then single cells were isolated. The cells were washed and resuspended in cold homogenizing buffer consisting of 250 mM-sucrose, 20 mM-Tris-HCl, 1 mM-dithiothreitol and 1 mM-Pefabloc (Roth, Karlsruhe, Germany; pH 7·4) and homogenized using ultrasound (Bandelin Electronics, Berlin, Germany). After centrifugation (16 000 ***g***, 60 min, 4°C), the supernatant was aliquoted

and frozen at −80°C until use. Total GST activity was determined spectrophotometrically at 340 nm and 30°C using 1-chloro-2,4-dinitrobenzene as substrate. Total protein content was measured using the method of Bradford with bovine serum albumin as standard protein.

Statistical evaluation

The new batch of cells for each experiment was isolated from a different donor. The cells were divided and were then treated with the test compounds. All experiments were conducted independently at least three times (three batches of cells). Means and standard deviations were calculated from at least three independently reproduced experiments. Data of at least three ($n \geq 3$) experiments were evaluated to establish two-sided significance levels of independently reproduced determinations. Differences due to different treatments were calculated with GraphPad Prism software version 4 (GraphPad Software Inc., San Diego, CA, USA) using one-way ANOVA with Dunnett's multiple comparison post-test or two-way ANOVA with Bonferroni's post-test. Where appropriate, Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA) was used to detect differences using unpaired *t*-test.

Results

Analysis of the fermentation supernatants

Table 1 shows the concentrations of SCFA in the supernatants used in the present study. In the SFS, the total SCFA amounted to 94·4 mmol/l whereas only 35·9 mmol/l were detected in the faeces control. Butyrate increased from 4 mmol/l in the faeces control to a concentration of 10 mmol/l in the inulin-derived fermentation sample.

Metabolic activity

In order to estimate the effect of the fermentation supernatant and of SCFA on viability of the primary colon cells, we measured kinetics of metabolic activity. For this, primary single cells could only be incubated for relatively short periods (<12 h) since pilot studies had shown that their viabilities (determined with trypan blue) decrease after 12 h from 89 (SD 3) % to 60 (SD 9) %. Table 2 shows relative values after setting the medium control to equal 100 % since the basic levels of metabolic activity varied highly between the different donors despite similar starting viabilities (90·7 (SD 5·6) %). Absolute values are shown in the footnote to Table 2 which shows that after 4 h of incubation with the test compounds, neither SFS, synthetic SCFA mix nor the faeces control impaired the cells' metabolic activities. Only the highest tested dose of butyrate (50 mM) significantly reduced metabolic activity (85·5 (SD 7·2) %) in comparison to the medium control, which was set to equal 100 %.

After 12 h of treatment, the metabolic activity was significantly increased by 15 % (v/v) SFS (123·5 (SD 5·4) %) and by 15 % (v/v) of the faeces control (133·8 (SD 20·4) %), indicating trophic effects due to the treatments. In contrast, corresponding concentrations of the SCFA mixture (15 %, v/v) and of butyrate (~1 mM) did not have these effects. Very high amounts of butyrate (50 mM) continued to significantly reduce the metabolic activity to 79·9 (SD 6·6) %, after 12 h, and further down to 67·0 (SD 9·1) % after 24 h. After the 24 h treatment period, the opposing effects of SFS which increased metabolic activity, and pure SCFA which had no effect, were still apparent and again pointed to trophic effects by the complex SFS on survival of primary cells. After 24 h of exposure, the faeces control was neither toxic nor trophic. Since the absolute values of metabolic activity in the medium controls at 4 h were decreased by 53·9 % after 12 h treatment and by 86·7 % after 24 h treatment, the complex SFS seems to compensate this loss of viability and retained metabolic activity on account of yet unidentified fermentation products.

RNA isolation and glutathione S-transferase gene expression

Gene expression was analysed using the human drug metabolism macroarray HS11 (Superarray) on which ninety-six genes for enzymes of the biotransformation are spotted on a nylon membrane. For standardization, 1 μg total RNA was used for each array. Twelve hours was the largest possible duration to incubate primary tissue *in vitro* and to recover sufficiently viable cells (76 (SD 15) %; trypan blue exclusion test) and enough intact RNA (7·6 (SD 2·8) μg/treated sample and 3·6–5·5 μg RNA/1 × 10^6 cells, respectively) to perform gene

Table 1. Concentration and molar ratios of the SCFA (mmol/l) in the fermentation sample determined using GC†

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Sample	Absolute and relative concentrations	Determined SCFA							
		Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Capronate	Total
SFS									
	mmol/l	68·3	14·3	0·0	10·9	0·5	0·4	0·0	94·4
	%	72·3	15·2	0·0	11·6	0·5	0·5	0·0	100·0
Faeces control									
	mmol/l	25·9	4·8	0·0	4·7	0·4	0·0	0·0	35·9
	%	72·3	13·4	0·0	13·1	1·2	0·0	0·0	100·0
Fold difference		2·6	3·0	–	2·3	1·0	–	–	2·6
SFS/faeces control		1·0	1·1	–	0·9	0·4	–	–	1·0

SFS, Synergy1 fermentation supernatant.
† Acetate, propionate and butyrate more than doubled after fermentation of Synergy1 whereas the ratio between the SCFA remained constant.

Table 2. Metabolic activity in human primary colon cells measured after 4, 12 and 24 h†
(Mean values and standard deviations)

	SFS‡		SCFA‡		Faeces§			Butyrate	
	4 h (%)								
Treatment (%)	Mean	SD	Mean	SD	Mean	SD	mM	Mean	SD
Medium	100	0·0	100	0·0	100	0·0	0	100	0·0
2·5	105	4·4	97	3·6	102	8·1	2	94	4·2
5	102	5·0	99	2·2	100	9·6	4	97	4·5
10	107	6·6	98	4·2	116	2·2	10	94	3·3
15	112	8·1	103	5·6	113	10·8	20	94	2·3
							50	85*	12·4
	24 h (%)								
Medium	100	0·0	100	0·0	100	0·0	0	100	0·0
2·5	113	5·3	108	8·4	113	13·5	2	109	7·1
5	115	10·6	102	20·5	116	10·6	4	101	10·9
10	115	7·0	108	5·0	122	16·3	10	107	7·5
15	124**	5·4	111	7·5	134*	20·4	20	97	4·6
							50	80*	6·6
	24 h (%)								
Medium	100	0·0	100	0·0	100	0·0	0	100	0·0
2·5	116	0·1	99	2·9	106	9·5	2	101	12·8
5	122*	4·1	110	13·9	105	4·3	4	94	9·5
10	128**[a,b]	11·4	94[a]	3·5	100[b]	9·5	10	86	11·8
15	127**	11·4	92	12·0	96	23·6	20	89	15·2
							50	67*	9·1

[a,b]Mean values with unlike superscript letters were significantly different ($P<0·05$; two-way ANOVA, Bonferroni's post-test).
Mean values were significantly different from those of the control (medium) group: *$P<0·05$; **$P<0·01$ (one-way ANOVA, Dunnett's multiple comparison test).
† The absolute metabolic activity of the controls decreased time-dependently from 8314 (SD 2372) fluorescence units (FU) to 3829 (SD 2257) FU after 12 h and to 1107 (SD 404) FU after 24 h in the medium controls (n 3).
‡ Concentrations of 10 % yielded treatment doses of 1·1 mM-butyrate and 9·4 SCFA in culture medium.
§ Concentrations of 10 % yielded treatment doses of 0·5 mM-butyrate and 3·6 mM SCFA (see also Table 1).

expression analysis. The same RNA was also used for confirmatory real-time quantitative PCR (qPCR) experiments.

The 'fold change' values were calculated from the normalized data. These were based on the corresponding values in the faeces control (complete fermentation supernatant) or the medium control (SCFA mixture). The changes were considered to be biologically significant if the ratio was $\leq 0·5$ or $\geq 2·0$ (Pool-Zobel *et al.* 2005*a*). Additionally, statistical significance was analysed using a two-sided t-test. Regulation by the SFS is summarized in Table 3 and a detailed presentation of all data on expression of GST in primary cells is shown in Table 4.

Table 3. Summary of those genes related to drug-metabolizing enzymes which were up- or down-regulated in primary colon cells treated with the fermentation sample in comparison to the faeces control†
(Mean values and standard deviations)

			Treatment of primary cells (12 h)				
			Faeces control		10 % SFS		
Functional gene family	Number of expressed genes		Mean	SD	Mean	SD	Fold change‡
Phase I, p450 family	7/25	*CYP7A1*	37·5	40·1	7·2	3·1	0·2
Phase II, glutathione *S*-transferases	9/12	*GSTA3*	35·0	40·6	14·5	6·3	0·4
		GSTM2	46·0	53·5	90·6	126·1	2·0
		GSTM5	62·3	63·2	140·1	194·9	2·2
Phase II, sulphotransferases	6/21	*SULT1A1*	18·6	14·4	54·5	36·6	2·9
		SULT1A2	12·8	13·6	60·1	45·8	4·7
Metallothioneins	8/8	*MT1G*	336·5	127·3	566·0	41·6	1·7§‖

SFS, Synergy1 fermentation supernatant.
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† Only those genes which reached an expression level with a signal over the cut-off level (≥ 15) in one of the treatments were evaluated (except the glutathione *S*-transferases, where all genes were considered).
‡ Fold changes $\geq 2·0$ and $\leq 0·5$ were considered as well as were those genes which are significantly different from the faeces control and as marked with symbols (§two-way ANOVA, Bonferroni's post-test; ‖unpaired t-test).

Table 4. Expression levels and modulation (fold change) of glutathione *S*-transferases by the Synergy1 fermentation supernatant (SFS) in comparison to the faeces control and in comparison to the medium control in primary colon cells†

(Mean values and standard deviations)

						Treatment of primary cells (12 h)												
	Faeces control		10 % SFS			Medium control		10 % faeces control			10 % SFS			10 mM-butyrate			10 % SynMix		
Functional gene family	Mean	SD	Mean	SD	Fold change	Mean	SD	Mean	SD	Fold change	Mean	SD	Fold change	Mean	SD	Fold change	Mean	SD	Fold change
GSTA2	47	46	25	12	**0·5**	22	10	47	46	**2·1**	25	12	1·2	44	30	**2·0**	29	15	1·3
GSTA3	35	41	14	6	**0·4**	14	8	35	41	**2·5**	14	6	1·0	27	21	**2·0**	15	7	1·1
GSTA4	7	5	6	1	0·9	3	4	7	5	**2·0**	6	1	1·7	9	7	**2·7**	4	4	1·3
GSTM2	46	53	91	126	**2·0**	200	310	46	53	**0·2**	91	126	**0·5**	199	309	1·0	83	116	**0·4**
GSTM3	32	15	35	32	1·1	50	43	32	15	0·6	35	32	0·7	67	73	1·3	32	13	0·6
GSTM5	62	63	140	195	**2·2**	272	419	62	63	**0·2**	140	195	**0·5**	342	543	1·3	206	312	0·8
GSTP1	123	64	118	25	1·0	117	20	123	64	1·1	118	25	1·0	168	133	1·4	98	34	0·8
GSTT1	5	3	6	6	1·2	7	5	5	3	0·6	6	6	0·8	6	5	0·7	10	4	1·3
GSTT2	141	94	154	46	1·1	136	34	141	94	1·0	154	46	1·1	276	237	**2·0**	106	40	0·8
MGST1	7	2	8	5	1·1	12	10	7	2	0·6	8	5	0·6	10	11	0·8	4	12	**0·3**
MGST2	25	4	30	7	1·2	35	7	25	4	0·7	30	7	0·9	42	30	1·2	30	13	0·9
MGST3	67	68	89	61	1·3	160	111	67	68	**0·4**	89	61	0·6	93	102	0·6	107	102	0·7

SynMix, synthetic SCFA mixture.

Q14 †The results for butyrate are already published in Pool-Zobel *et al.* (2005*b*). All genes of this family were evaluated. Mean changes (n 3) with a fold change ≥2·0 and ≤0·5 were considered to be different from the medium control. The genes which met these criteria are written in bold.

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There were large variations in the levels of gene expression between the different donors for several of the analysed genes. The changes due to treatments, however, pointed to many of the genes in the same direction. Otherwise, they were not considered for additional assessment.

Seven genes were altered by the fermentation supernatant in comparison to the faeces control. CYP7A1 was reduced whereas two sulphotransferases were enhanced. Expression of MT1G was also increased. Table 4 shows the differential expression of GST genes based on the faeces control. It is apparent that *GSTM2* and *GSTM5* were up-regulated whereas there was a clear reduction of *GSTA2* and *GSTA3* expression. The expression of *GSTM2* and *GSTM5* were subjected to confirmatory analysis by real-time qPCR. *GSTA4* was additionally included as a negative control. Real-time qPCR analysis confirms the directional changes of the macroarray since *GSTM2* was induced 1·4-fold (2-fold in the array) and *GSTM5* 2·0-fold (2·2-fold in the array). The 'negative' control *GSTA4* which was not induced according to the array analysis (0·9-fold) was also not altered according to real-time qPCR (1·0-fold).

The comparison of the faeces control (Table 4) to the medium control also shows that a number of genes were differentially regulated. There was, for instance, a down-regulation of *MGST3* (0·4-fold), *GSTM2* and *GSTM5* (each 0·2-fold), but a marked up-regulation of *GSTA1*, *GSTA2* and *GSTA4* (2–2·5-fold) by the faeces control. The responsible factors are probably certain bacterial metabolites (other than SCFA) which, however, have not yet been identified. According to real-time qPCR analysis there was also an induction of *GSTA4* (1·3-fold) compared to the medium control, but the decreases of *GSTM2* (0·8-fold) and *GSTM5* (0·9-fold) were not as strong as observed in the array analysis (Fig. 1). From Table 4 it is apparent that the SFS when compared to the medium control seems to counteract the *GSTM2*, *GSTM5* and *MGST3* reduction, but is not as efficient in enhancing *GSTA*s.

Confirmatory analysis of the up-regulation of *GSTA2* and *GSTA3* by the faeces control in comparison to the medium control could not be performed. Due to high homology between the A1, A2 and A3 isoforms, the available GSTA2 and GSTA3 cDNA primer sequences were not specific enough to yield specific PCR products.

Table 4 also compares the effects of the synthetic SCFA mixture to previously published data on butyrate (Pool-Zobel *et al.* 2005*a*). When compared to the medium control the butyrate treatment of primary colon cells results in an up-regulation of *GSTA2*, *GSTA3*, *GSTA4* and *GSTT2*, whereas the SCFA mixture seems to down-regulate *GSTM2* and *MGST1*. This indicates that the SCFA combination (delivering ~10 mM-butyrate, acetate and propionate to the cell suspension) is far less effective than butyrate (10 mM). The respective confirmatory analysis for butyrate treatment was performed for *GSTA4* and *GSTT2* and for the moderately altered *GSTP1* since these had been studied in the macroarray analysis (2·7-, 2·0- and 1·4-fold, respectively), but had not previously been confirmed. Fig. 1 shows that these changes in expression levels by butyrate were largely confirmed by real-time qPCR. Expression results for *GSTM2* and *GSTM5* after treatment with the SCFA mixture were confirmed (0·4- and 0·8-fold in the microarray, respectively). Fig. 1 shows that the results for *GSTA4* also confirm the data of the macroarray analysis, whereas the reduction of *GSTM2* was not detected with real-time PCR.

Quantification of total glutathione S-transferase activity

GST activity was measured as a functional characteristic of gene expression using 1-chloro-2,4-dinitrobenzene as substrate. A number of GST isoenzymes utilize this compound as substrate and are capable of catalysing the conjugation reaction. In particular, there was a distinct correlation between GSTP1 protein expression and GST activity in HT29 colon cells (Ebert *et al.* 2003). There was already a loss of basal GST activity in primary colon cells treated only with medium for 12 h. The initial activity of 211 (SD 79) nmol/min per mg protein was reduced to 146 (SD 77·6) nmol/min per mg protein after this period. The treatment of colon cells with the different test compounds slightly reduced the enzyme activity even more, as shown in Table 5. Both complex samples (SFS and faeces blank) were inhibitory resulting in only 73 (SD 12) and 81 (SD 6) % of the activity in the medium control which was set to 100 %. In comparison, the SCFA mixture and butyrate were less inhibitory. When compared to the faeces control (which was set to equal 100 %),

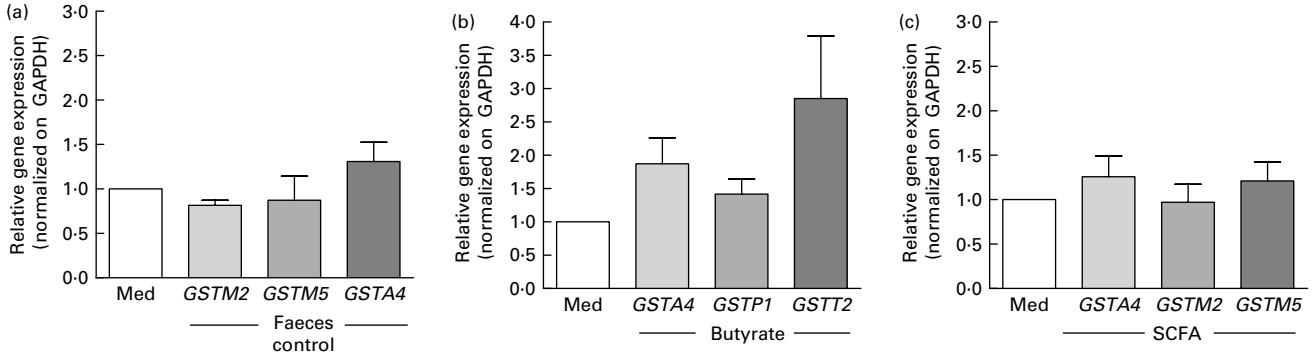

Fig. 1. Quantitative analysis of glutathione *S*-transferase (GST) mRNA by real-time PCR in primary cells treated with the faeces control (a), butyrate (b) and the SCFA mixture (c) for 12 h. We present here results also for the butyrate treatment verifying array results published in Pool-Zobel *et al.* (2005*b*). Values are means with their standard deviations depicted by vertical bars (n 3). The relative gene expression analysis showed a 1·9-fold up-regulation for *GSTA4*, a 2·9-fold increase for *GSTT2* (n 6) and a 1·4-fold change for *GSTP1* (n 3) after butyrate treatment compared to medium-treated (Med) control cells (unpaired t-test, Welch's correction). GADPH, glyceraldehyde-3-phosphate dehydrogenase.

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GST activity was hardly altered by the SFS. However, the observed differences were not significant.

Discussion

Inulin enriched with oligofructose (Synergy1) is a source of dietary fibre which yields high amounts of butyrate due to fermentation by gut bacteria. Here, the *in vitro* fermentation of inulin enriched with oligofructose almost tripled the concentration of SCFA in the supernatant. These *in vitro* concentrations of SCFA in the SFS correspond to absolute amounts and molar ratios found after high consumption of dietary fibre *in vivo* (Beyer-Sehlmeyer *et al.* 2003). Several *in vitro* and *in vivo* rat studies have shown that inulin or the complex fermentation products may exert protective effects in colon cells. Since so far only little is known in non-transformed human colon cells we have developed techniques to measure a modulated state of gene expression and functional consequences leading to chemoprotection in normal colon cells.

As shown in previous studies, butyrate and complex fermentation supernatants are potent inhibitors of cell growth in colon tumour cells. Here the results show that primary human colon cells tolerated butyrate and fermentation supernatants in high amounts during short-term incubations. The loss of metabolic activity throughout the experiments was time dependent and was barely influenced by increasing concentrations. The increase of metabolic activity by the SFS after 12 and 24 h pointed to nutrient effects and indicated that the remaining cells were more active after treatment with the SFS. Only butyrate reduced metabolic activity at 50 mM in the tissue specimens used in the present study. This effect may be donor-specific since the same treatments in specimens of other donors (n 3) indicated trophic effects (increased metabolic activity) at 50 mM after 4 h (J Sauer, KK Richter & BL Pool-Zobel, unpublished results).

The results from the metabolic assay and from the gene array analysis revealed that undefined compounds in the faeces supernatant also have effects on the cells. These factors could include bile acids (e.g. lithocholic acid and desoxycholic acid) and other unidentified bacterial products (Roberfroid, 2005). The compounds seem to suppress cell growth in tumour and adenoma cell lines since SFS were more active for inhibiting survival of cell lines than butyrate alone (J Kiefer & D Scharlau, unpublished results). In contrast, the same SFS enhanced metabolic activity in the primary cells, pointing to nutritive effects by products formed during the fermentation of inulin in non-transformed primary cells. Moreover, this normal cell type seemed to be less susceptible to the treatment with the fermentation supernatant than colon cancer cell lines.

Butyrate, as one of the main active fermentation products, is known to modulate gene expression. Previously, we were able to show that important enzymes of biotransformation are inducible in colon cell models. For instance, butyrate can enhance the expression of GST in human colon tumour cells (Ebert *et al.* 2001, 2003; Pool-Zobel *et al.* 2005*a*). The present study investigated the potential of fermentation products derived from inulin to modulate genes related to drug metabolism in primary human colon cells. This is considered to be an important mechanism leading to chemoresistance of the affected cells. Altogether, the treatment with the SFS in comparison to the faeces control altered seven genes. SFS induced *GSTM2* and *GSTM5* which are capable of detoxifying electrophilic compounds that include carcinogens or environmental toxins. Additionally, these GSTM isoforms possess peroxidase activity (Hayes & Strange, 2000; Hayes *et al.* 2005). Moreover, products of oxidative stress can be quenched by conjugation with glutathione. Expression of *CYP7A1* was lowered which might result in a reduced metabolic activation of xenobiotics by monooxygenases. In the liver, CYP7A1 plays a key role in cholesterol degradation. It binds cholesterol and converts it to 7α-hydroxycholesterol (Mast & Pikuleva, 2005). Thus, CYP7A1 primarily regulates the pathway through which cholesterol is converted into bile acids. Two sulphotransferases, SULT1A1 and SULT1A2, were enhanced. Sulphate conjugation is an important pathway in the biotransformation of many exogenous and endogenous compounds. These enzymes catalyse sulphate conjugation of many phenolic or catechol drugs and other xenobiotics as well as endogenous compounds (e.g. oestrogens; Carlini *et al.* 2001) and can therefore enhance the cellular detoxification capacities. But it is also described that sulphation can result in activation or metabolic activation of sulphate acceptor substrates (Falany, 1997). These functional consequences (good or bad) will depend on types of exposure that occur. The induction of *MT1G*, however, might mainly be a favourable effect since this family of metal-binding proteins can scavenge metal ions, free radicals, toxins and activated xenobiotics (Coyle *et al.* 2002). Altogether, the observed shift in gene expression patterns could be possibly related to chemoprevention since by enhancing the detoxification capacity it is possible to reduce exposure to carcinogenic compounds.

The faeces blank was also able to alter gene expression when compared to the medium control, again pointing to yet unidentified factors in the faecal matrix that are responsible. The changes in gene expression by the SFS are not only attributable to the content of SCFA since the synthetic mixture did not cause the same effects. The SCFA combination was less effective than butyrate (Pool-Zobel *et al.* 2005*a*) which could be due to concentration-related effects. The mixture contained only approximately 1 mM-butyrate, but approximately 10 mM-total SCFA. The difference in effects is

Table 5. Glutathione *S*-transferase (GST) activity after 12 h treatment with the fermentation product and corresponding controls

(Mean values and standard deviations)

	GST activity (nmol/min per mg protein)		Relative GST activity (%)†		Cell viability (%)‡	
	Mean	SD	Mean	SD	Mean	SD
Medium	146	77·6	100	0	69	11
SCFA	106	28·7	85	37	61	16
Butyrate	116	57·8	88	34	71	13
Faeces control	154	36·6	100	0	50	15
SFS	140	46·2	90	9	68	5

SFS, Synergy1 fermentation supernatant.

† Relative total GST activity was calculated separately for each experiment.

‡ Cell viability decreased in comparison to the basal viability of 90 (SD 4) %, but there were no significant differences between the treatments and the medium control (n 3, one-way ANOVA, Dunnett's multiple comparison test).

therefore possibly based on combination effects. Alternatively, the detected effects are only attributable to butyrate. Butyrate itself can directly act on gene expression because of butyrate responsive factors (e.g. butyrate responsive factor 1) on promoters (Maclean *et al.* 1998; Patel *et al.* 2005) or as a potent histone deacetylase inhibitor via histone deacetylation (Boffa *et al.* 1992). Since butyrate is oxidized by the colonocytes its metabolites may play a role in exerting genetic effects, but the role of its metabolites is still unexplained.

In the study presented here, there was also a pronounced variation of expression levels, e.g. of the GSTM isoforms between different donors, which we were not able to explain by diagnosis, gender or determined GST polymorphisms.

Moreover, we found a reduction of GST activity after 12 h treatment, and the lowest values were observed in cells treated with the faeces blank and SFS. This reduction may reflect the decrease of *GSTM2* and *GSTM5* on transcriptional level. The induction of GSTA isoforms which have only a moderate affinity for 1-chloro-2,4-dinitrobenzene (Eaton & Bammler, 1999), and which are not abundant in the colon cells, would probably not be detected by the GST activity measurement. In contrast, butyrate tended to induce GST gene expression, but this could also not be confirmed by the measurement of GST activity. The reasons for this may be that one of the most induced GST forms, namely GSTT2, does not use 1-chloro-2,4-dinitrobenzene as a substrate (Eaton & Bammler, 1999). Another reason may be that the 12 h incubation was too short to detect changes in protein expression levels.

Other *in vitro* studies in HT29 cells also did not show that a complex fermentation sample (produced from different dietary fibre sources) induced GST activity which was explained by too low butyrate concentrations and by the presence of other inhibitory substances in the faeces (Beyer-Sehlmeyer *et al.* 2003). GST activity, however, was enhanced in HT29 human colon cells after 72 h treatment with the major fermentation product butyrate (Ebert *et al.* 2001). This supports the conclusion that the butyrate concentration in the fermentation supernatants is responsible for effects in cellular systems although other compounds like propionate could additionally contribute to selected activities (Beyer-Sehlmeyer *et al.* 2003).

All in all, the present experiments substantiate the hypothesis that butyrate and complex fermentation supernatants may result in favourable effects in non-transformed colon cells. Ongoing studies will lead to a better understanding on how inulin-type fructans and the resulting fermentation supernatants can affect primary colon cells by modulating gene expression and how these changes are connected with a protection from colon cancer development.

Conclusions

The present studies have shown that fermentation products derived from inulin enriched with oligofructose favourably modulated profiles of genes related to xenobiotic metabolism in primary human colon cells. The cells retained more of their metabolic activity than cells not treated with the sample which implies the presence of compounds with nutrient functions. The present studies support the decisive role of butyrate in terms of modulating gene expression (Pool-Zobel *et al.* 2005*a*) in primary healthy human colon cells, but the butyrate concentrations were possibly only suboptimal in the fermentation supernatant as investigated here. The faeces samples also had effects on metabolic activity and gene expression with high interindividual variability. It will be an important goal in the future to find the underlying factors responsible for the variation. The present study provides insight into how fermentation products of dietary fibre, particularly butyrate, can affect non-transformed primary human colon cells. It allows the conclusion that the fermentation products of inulin might have chemopreventive activities in non-transformed human colon cells.

Note

Q8 Supplementary information accompanies this paper on the Journal's website (http://www.nutritionsociety.org) (Sup-
Q7 plementary Table 1).

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Supplementary Table 1. Summary of those genes related to drug metabolizing enzymes which were up- or down-regulated in primary colon cells treated with the test samples in comparison to the medium control. The cells were treated for 12 h with the fermentation supernatant derived from Synergy and human faeces. Synthetic mixtures of SCFA (composed to mimic the complex SFS), medium or faeces controls were investigated in parallel. Only those genes which reached an expression level with a signal over the cut off level (≥15) in one of the treatments were evaluated (except the GSTs, where all genes were considered). Fold changes ≥2·0 and ≤0·5 were considered in the evaluation as were those genes which are statistically significantly different from the medium, as are marked with symbols (* = two way ANOVA, Bonferroni's post-test; § = unpaired t-test; # = fold change calculation).

			Treatment of primary cells [12 h]							
			Medium control		10 % SynMix		10 % SFS		10 % Faeces control	
Functional gene family	Number of expressed genes		Means	SD	Up	Down	Up	Down	Up	Down
Phase, I p450 Family	7/25	CYP7A1	17	12				CYP7A1 #	CYP7A1#	
Phase II, Acetyltransferases	8/10	ACAT2	840	236				ACAT2***		ACAT2***
		DLAT	17	13						DLAT#
Phase II, Glutathione *S*-Transferases	9/12	GSTA2	22	10					GSTA2#	
		GSTA3	14	8					GSTA3#	
		GSTM2	200	310		GSTM2 #		GSTM2#		GSTM2#
		GSTM5	272	419						GSTM5#
		MGST3	160	111						MGST3
Phase II, Sulfotransferases	6/21	SULT1A1	13	14			SULT1A1#			
		SULT1A2	16	6			SULT1A2#			
		CHST7	18	26		CHST7#				
Phase II, Miscellaneous	6/13	HNMT	169	156		HNMT#		HNMT#		HNMT #
Phase III, Metallothioneins	8/8	MT1G	632	336						MT1G**
		MTIX	698	271						MTIX**
Phase III, p-Glycoproteins	3/7									

2.4 Publikation IV: PHYSIOLOGICAL CONCENTRATIONS OF BUTYRATE FAVORABLY MODULATE GENES OF OXIDATIVE AND METABOLIC STRESS IN PRIMARY HUMAN COLON CELLS.

Julia Sauer, Konrad K. Richter, Beatrice L. Pool-Zobel. *Journal of Nutritional Biochemistry.* Angenommen am 06.12.2006.

Eine Steigerung der Expression von antioxidativen und eine Senkung von inflammatorischen Enzymen könnten zur Chemoprävention im Kolon beitragen. In dieser Studie wurde das Vermögen von Butyrat untersucht, die Expression derartiger Enzymsysteme in primären, nicht-transformierten Kolonzellen zu verändern. Hierzu wurden ein Stoffwechselweg-spezifischer Gen-Array, real-time PCR für ausgewählte Gene und Katalase-Aktivitätsmessung angewendet. Die Butyratbehandlung primärer Zellen beeinträchtigte die Vitalität nicht. Über eine Zeitdauer von 12 h wurden 0,3 µmol Butyrat je $1x10^6$ Zellen aufgenommen. Die Expression von Katalase und COX-2 konnte durch Butyrat verändert werden, wobei starke interindividuelle Variationen zwischen den Einzelspendern auftraten. Eine gesteigerte Katalase-Aktivität könnte die Exposition gegenüber H_2O_2 verringern, während eine Abnahme der COX-2 Expression inflammatorische Prozesse reduzieren könnte. Durch diese Modulationen könnten Zellen vor Schädigungen durch Peroxide oder reaktive Sauerstoffspezies geschützt werden, was die Initiation des Transformationsprozesses verhindern könnte.

Eigenanteil:

- Isolierung und Aufarbeitung der Kolongewebeproben
- Messung der Vitalität und metabolischen Aktivität
- Durchführung der Inkubationen für die Butyrataufnahme
- RNA-Isolation und Durchführung der Gen-Arrays sowie Durchführung der real-time PCR und Messung der Katalase-Aktivität
- Auswertung, Interpretation und Darstellung der Ergebnisse
- Verfassung des Manuskriptes

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Physiological concentrations of butyrate favorably modulate genes of oxidative and metabolic stress in primary human colon cells☆

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Abstract

Butyrate, a metabolite of gut flora-mediated fermentation of dietary fibre, was analysed for effects on expression of genes related to oxidative stress in primary human colon cells. An induction of detoxifying, antioxidative genes is expected to contribute to dietary chemoprevention. Cells were treated with butyrate (3.125–50 mM; 0.5–8 h), and kinetics of uptake and survival were measured. Gene expression was determined with a pathway-specific cDNA array after treating colon epithelium stripes with nontoxic doses of butyrate (10 mM, 12 h). Changes of *hCOX-2*, *hSOD2* and *hCAT* expression were confirmed with real-time polymerase chain reaction (PCR) and by measuring catalase-enzyme activity. Primary colon cells consumed 1.5 and 0.5 mM butyrate after 4- and 12-h treatment, respectively. Cell viability was not changed by butyrate during 0.5–2-h treatment, whereas cell yields decreased after 1 h. Metabolic activity of remaining cells was either increased (4 h, 50 mM) or retained at 97% (8 h, 50 mM). Expression of *hCAT* was enhanced, whereas *hCOX-2* and *hSOD2* were lowered according to both array and real-time PCR analysis. An enhanced catalase-enzyme activity was detected after 2 h butyrate treatment. Healthy nontransformed colon cells well tolerated butyrate (50 mM, 2 h), and lower concentrations (10 mM, 12 h) modulated cyclooxygenase 2 (COX-2) and catalase genes. This points to a dual role of chemoprotection, since less COX-2 could reduce inflammatory processes, whereas more catalase improves detoxification of hydrogen peroxide (H_2O_2), a compound of oxidative stress. Changes of this type could reduce damaging effects by oxidants and protect cells from initiation.

Keywords: Butyrate; Human colon cells; Oxidative stress; Gene expression

1. Introduction

Colorectal cancer remains one of the major health problems and is the second most common cause of death due to cancer in countries with Western style diets [1]. Epidemiological and animal studies suggest that a diet high in fat, red meat and protein may increase the risk of colon cancer, whereas a high intake of fibre and complex carbohydrates may be protective [2,3].

There are some controversial findings in other epidemiological surveys either negating the protective roles of fibre [4,5] or the causative role of red meat [6]. By and large, however, most experimental studies directed at elucidating molecular, toxicological and chemoprotective associations support the roles of fibre in chemoprevention or of meat in posing a risk. In extension of these epidemiological findings, experimental studies suggest that a diet high in red meat might pose a risk on account of the iron contained in the heme [7,8]. The iron of heme or haemoglobin can, for example, catalyse the formation of reactive oxygen species that contribute to colon cancer development by inducing genotoxic damage [8]. Alternatively, heme from red meat can also increase endogenous *N*-nitroso compounds in the faeces, which has been positively correlated with the formation of alkylating DNA adducts like *O*(6)-carboxymethyl guanine in exfoliated colon cells [9]. In vivo, it seems feasible that such reactive compounds damage the colon crypt cells, resulting in initiation or in an enhanced progression of initiated cells [10]. Indeed, it has been shown that the faecal matrix itself is also capable of generating reactive oxygen species and acting genotoxic after a diet high in meat and low in fibre [11,12].

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One mechanism of chemoprevention could involve protection against genotoxic compounds at the preinitiation stage [13,14]. Thus, it has been shown that colon cells contain several compounds which can capture free radicals. Moreover, the cells express different types of detoxifying and stress-response enzyme systems [15], including glutathione *S*-transferases (GSTs), peroxidases and catalase. GSTs are known to be phase II enzymes that can detoxify a number of carcinogens, and some have clear peroxidase activity [16]. Catalase is one of the key defense systems against oxidative stress since it very rapidly detoxifies H_2O_2 to yield H_2O and O_2. Therefore, a high level of expression could be associated with less genetic damage due to the exposures that involve colorectal cancer risk [17].

A basic preliminary finding has shown that a short-term preincubation of primary colon cells of rats and humans with butyrate significantly reduced the genotoxic effects of H_2O_2 [18,19]. Nothing, however, is known on the potential mechanisms behind this effect. Since butyrate, a product formed in millimolar concentrations during gut fermentation of dietary fibres is able to modulate gene expression in human colon cells [20], e.g., via inhibition of histone deacetylases or via genomic response elements [21], it is now hypothesized that butyrate could enhance expression of genes that reduce oxidative and metabolic stress [20].

Another risk factor for the development of colon cancer is inflammation. It is known that, in colon tumors, cyclooxygenase 2 (COX-2) is overexpressed, which increases formation of inflammatory prostaglandins. Since inflammatory processes may also result in oxidative stress and generate free radicals, inhibition of COX-2 is possibly an effective mode of cancer chemoprevention [22].

The aim of this study was to find out whether expression of genes involved in these pathways of stress and toxicity (GSTs, peroxidases, catalase, COX-2) can be changed by physiologically relevant concentrations of butyrate in human primary colonocytes. In extension of this, it was a further aim to assess whether these changes result in functional consequences that could explain the previously observed reduction of H_2O_2 genotoxicity in butyrate-pretreated nontransformed colon cells. These mixed cell suspensions contain also the actual target cells of colon carcinogenesis (stem cells and dividing daughter cells [23]). It is, however, technically challenging to work with these cells in vitro since they have only a limited life span after isolation [24,25].

We present new findings on effects of butyrate in healthy nontransformed colon cells and report that genes associated with oxidative stress can be favorably modulated by this important short chain fatty acid.

2. Methods and materials

2.1. Primary colon tissue preparation and isolation of cells

The study was approved by the ethics committee of the Friedrich–Schiller University of Jena, and patients gave their informed consent. Primary human colon cells were isolated from tissue specimens obtained during surgery of colorectal tumors, diverticulitis and colon polyps (Table 1). The tissues were taken from the very edges of the resected colon segments. The surgeon and the pathologist confirmed that they did not show any microscopic or macroscopic signs of malignant or inflammatory pathology. Table 1 summarizes all diagnoses that were the basis for performing each surgery. Stenotic anastomosis was a reason for one of the surgical procedures. The anastomosis of this patient was constricted and, thus, made it necessary to resect the colon

t1.1 Table 1
t1.2 Summary of the available information about the donors whose cells were used for the different experiments

t1.3 Donor	Gender (male/female)	Age (years)	BMI (kg/m^2)	Diagnosis	Used in experiment
t1.4 1	F	40	20.8	Anastomosis stenosis	Array, real-time PCR
t1.5 2	M	70	25.8	Adenoma	Array, real-time PCR
t1.6 3	F	52	25.4	Sigma carcinoma	Array, real-time PCR
t1.7 4	M	57	23.9	Sigma diverticulitis	Real-time PCR
t1.8 5	M	64	24.2	Sigma diverticulitis	Real-time PCR
t1.9 6	M	53	30.3	Sigma carcinoma	Real-time PCR
t1.10 7	M	62	28.7	Sigma carcinoma	Viability, cell number, metabolic activity
t1.11 8	F	77	25.0	Morbus Crohn	Viability, cell number, metabolic activity
t1.12 9	M	63	31.6	Sigma diverticulitis	Viability, cell number, metabolic activity
t1.13 10	F	66	48.8	Rectum carcinoma	Butyrate uptake
t1.14 11	M	42	23.5	Sigma diverticulitis	Catalase activity
t1.15 12	M	58	18.9	Colon carcinoma	Catalase activity, butyrate uptake
t1.16 13	M	37	24.1	Rectum carcinoma	Catalase activity
t1.17 14	F	54	27.3	Sigma carcinoma	Catalase activity
t1.18 15	M	50	20.2	Rectum carcinoma	Butyrate uptake
t1.19 16	F	52	23.1	Sigma diverticulitis	Butyrate uptake
t1.20 17	M	68	23.7	Rectum carcinoma	Butyrate uptake
t1.21 18	M	68	25.3	Rectum carcinoma	Catalase activity
t1.22 19	F	59	23.0	Sigma carcinoma	Catalase activity
t1.23 Mean±S.D.	(12/7)	57±11	26±6		

t1.24 Mean age of the patients was 57 years, 12 were male and seven were female.

section, but the tissue was confirmed by histopathology to be nonmalignant, and the experimental slices were isolated from the edges of the resected bowel segment. Mean age (+S.D.) of the donors of colon cells for the experiments (metabolic activity, RNA isolation, cytosol preparation, butyrate consumption studies) was 57+11 years; 12 of the donors were male, seven were female. The tissue was stored in Hank's balanced salt solution (HBSS) (8.0 g/L NaCl; 0.4 g/L KCl; 0.06 g/L $Na_2HPO_4 \times 2\ H_2O$; 0.06 g/L K_2HPO_4; 1 g/L glucose; 0.35 g/L $NaHCO_3$; 4.8 g/L HEPES; pH 7.2), transported on ice to the laboratory within 1 h and worked up immediately. The human colon epithelium was separated from the tissue by perfusion-supported mechanical disaggregation [26].

Subsequently, single cells were isolated from the epithelial stripes by mincing and were incubated in 3 ml HBSS (60 min, 37°C) supplemented with 6 mg proteinase K (Sigma; Steinheim, Germany) and 3 mg collagenase P (Boehringer; Mannheim, Germany). The suspensions of primary human colon cells were diluted with HBSS, centrifuged and resuspended in phosphate-buffered saline (PBS) (8 g/L NaCl; 1.44 g/L Na_2HPO_4; 0.2 g/L KCl; 0.2 g/L KH_2PO_4; pH 7.3). Single cells were seeded into wells of 96-well microtiter plates and treated as indicated in tables and figures. Alternatively, intact tissue stripes were treated with butyrate up to 12 h for gene expression studies. Viability and cell yields were determined with trypan blue before performing the metabolic activity assay and before isolating RNA.

2.2. Quantification of butyrate uptake by gas chromatography

The concentration of butyrate in culture supernatants of butyrate-treated primary human cells was measured by gas chromatography using a GC17-A gas chromatograph (Shimadzu, Duisburg, Germany) [27,28]. For this, 5×10^6 cells were treated with 10 mM butyrate, which was dissolved in 3 ml cell culture medium. The culture supernatants, collected after 2, 4, 8 and 12 h of incubation, were stored at −20°C and were analysed for the remaining butyrate concentration, as described previously [29]. The difference between the concentration before and after the treatment is estimated to represent the amount of butyrate which is intracellular available. The samples were thawed and centrifuged to remove cellular components. Fifty microliters of isocaproic acid (0.89 μg/ml in concentrated formic acid; internal standard for calibration) were added to 0.5 ml of each sample of which 1 μl was injected and evaporated at 250°C. The internal standard isocaproic acid was the basis for calculating the butyrate concentrations.

2.3. Determination of viability, cell number and measurement of metabolic activity

Cell suspensions containing 2×10^6 cells/ml were incubated for 0.5–4 h, with 0–50 mM butyrate in a shaking thermomixer at 37°C. The trypan blue exclusion test was routinely used to determine cell viability and cell number after incubating the cells in suspension with butyrate.

For the metabolic activity assay, isolated cells were seeded into 96-well microtiter plates (50 000 cells per well) and incubated in minimal essential medium with Earle's salts enriched with 20% FCS, 2 mM glutamine, 1% penicillin/streptomycin, 100 μg/ml gentamycin, 2.5 μg/ml fungizone, 10 ng/ml EGF, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite [30].

To determine biological effects of physiological concentrations of butyrate (0–50 mM), the primary colon cells were incubated for 2, 4, 6 and 8 h. Metabolic activity as a surrogate parameter of cytotoxicity was assessed using the CellTiter-Blue assay (Promega, Mannheim, Germany) in 96-well microtiter plates with measurements after different time points. The CellTiter-Blue assay is a viability assay, which can be used to estimate the number of viable cells in nonadherent cell suspensions, such as primary colon cells. To measure the metabolic capacity of the cells, this assay uses the dye resazurin, which is reduced into resorufin only by viable cells. This product is highly fluorescent and was detected with Ex/Em 520/595 nm after 2 h incubation with the reagent. Mean values were calculated from means of three parallel determinations of three independent experiments.

2.4. Treatment with butyrate for gene expression analysis

Effects of butyrate on gene expression were studied after incubating the epithelial tissue stripes with 10 mM butyrate instead of using single cells, since this results in an improved survival of the target cells for up to 12 h [20]. Primary human colon tissue pieces were plated in Petri dishes (35 mm) and, after allowing the tissue pieces to settle for 15 min, they were treated with 10 mM butyrate-dissolved cell culture medium, as described in Section 2.3 [30]. After 12 h, single cells were isolated, as described above, quantified and further processed for RNA isolation.

2.5. RNA isolation

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 50 μl RNase-free water and stored at −20°C. The ratio $A^{260}/_{280}$ and the concentration of total RNA was determined spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany) for protein or phenol contamination. This was followed by formaldehyde denaturing RNA gel electrophoresis to check the integrity of the ribosomal RNA and DNA contamination. Contaminating DNA was eliminated by DNase-I treatment using the RNase-free DNase I Amplification Grade kit (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. Subsequently macro array analysis was carried out.

2.6. Macroarray analysis

Hybridization was performed on 112 genes (three blanks, 13 reference spots and 96 human genes related to stress and toxicity) on cDNA gene macroarrays (GEArray Q Series Human Stress&Toxicity Gene Array HS12; SuperArray

Bioscience, Frederick, MD, USA). Genes were classified into functional categories, representing genes belonging to Proliferation/Carcinogenesis, Growth Arrest/Senescence, Inflammation and Necrosis/Apoptosis (Oxidative & Metabolic Stress, Heating Stress, DNA Damage & Repair and Apoptosis Signaling). A detailed gene list is available at the company's Web site (www.superarray.com). Workup of the array was performed according to the manufacturer's protocol and as has been previously reported for another array type from the same company [20]. Briefly, single-stranded cDNA was synthesized from total RNA (1 μg) in vitro. By applying a single-step ampo linear polymerase reaction technique, the cDNA was labelled with dUTP-biotin. The cDNA macroarray was hybridized overnight at 60°C with the biotin-labelled cDNA. The hybridized membrane was subjected to chemiluminesence analysis for quantification of the conjugation signals with streptavidin-linked alkaline phosphatase and CDPstar. The resulting signals were captured with a CCD camera (Fujifilm LAS-1000, Diana, USA) and analyzed with AIDA array analysis (Raytest GmbH, Germany) software to evaluate the differential gene expression of the various samples. Raw data were normalized between 0% and 100% expression, where the signals of the means of the negative controls (areas without spotted gene sequences or with genes not expressed in human cells) were set to equal 0% and the means of the signals of the positive controls (household genes) were fixed to equal 100%. Thus, the data shown here represent mean expression levels relative to negative and positive reference genes.

2.7. Determination of hCOX-2, hSOD2 and hCAT expression with real-time polymerase chain reaction in primary colon cells

The expression of *hCOX-2*, *hSOD2* and *hCAT* was verified by quantitative real-time polymerase chain reaction (PCR) using the system of iCycler iQ (Biorad GmbH, München, Germany). One microgram of total RNA was subjected to reverse transcription (SuperScript II, First-Strand cDNA Synthesis System; Invitrogen) in 20 μl buffer with oligo $(dT)_{15}$ primers, according to manufacturers instructions. Fifty nanograms of cDNA, calculated as RNA equivalents, were used in a 25 μl PCR amplification reaction containing 2x iQ SYBR Green supermix (100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, stabilizers) and 10 pmol gene-specific primers for the target genes and reference (*hGAPDH*) gene. The following primer sequences were used to amplify a region of *hCOX-2*, *hSOD2* and *hCAT* and *hGAPDH* mRNA:

COX-2-F (5′ -tcc tcc tgt gcc tga tga ttg c-3′)
COX-2-R (5′ -act gat gcg tga agt gct ggg-3′)
CAT-F (5′ -tgg aca agt aca atg ctg ag-3′)
CAT-R (5′ -tta cac gga tga acg cta ag-3′)
SOD2-F (5′ -gcc ctg gaa cct cac atc aac-3′)
SOD2-R (5′ -caa cgc ctc ctg gta ctt ctc-3′)
GAPDH-F (5′ -cca ccc atg gca aat tcc atg gc-3′)
GAPDH-R (5′ -agt gga ctc cac gac gta ctc ag-3′).

PCR cycles included 1 cycle of 95°C for 2 min, followed by 40 cycles each of 94°C for 30 s, annealing temperature of 57°C for 30 s and 72°C for 40 s and a final extension step of 72°C for 10 min. Product-specific amplification was confirmed by melting curve analysis and agarose gel electrophoresis. All experiments were performed in duplicates. The fluorescence threshold value (C_T) was calculated using the iCycler iQ optical v3.0a system software. The relative quantification of the target-*mRNA* expression was calculated with the comparative $\Delta\Delta C_T$ ($\Delta C_T = \Delta C_{T\,\text{control}} - \Delta C_{T\,\text{reference}}$) method. For normalization, ΔC_T values were calculated by subtracting the average of the C_T value in the control for the reference gene from the average of the C_T value for the target gene and subtracting the average of the C_T value in the treated sample of the reference gene from the target gene. Then, the difference between the ΔC_T values of control and treatment ($\Delta\Delta C_T$) was calculated. The fold change was calculated according to the efficiency method ($E = 2$; fold change $= E^{\text{difference}}$) [31,32].

2.8. Preparation of cytosol, measurement of cytosolic protein and catalase activity

After incubation with butyrate, the cells were washed with PBS and then resuspended and lysed in cold phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% Triton-X. After centrifugation (10000*g*, 10 min, 4°C), the supernatant was aliquoted and frozen at −80°C until use. Total protein content was measured using the method by Bradford with bovine serum albumin as standard protein [33]. Catalase activity was assayed spectrophotometrically at 25°C by following the extinction of H_2O_2 at 240 nm by the method of Aebi [34]. Assay mixtures contained 10 mM H_2O_2 and 100 μl of cell lysates in 50 mM potassium phosphate buffer (pH 7.0). Enzyme activities were calculated using 0.0394 $mM^{-1} \times cm^{-1}$ as absorption coefficient at 240 nm.

2.9. Statistical analyses

GraphPad Prism software version 4 (GraphPad Software, San Diego, CA, USA) was used to calculate one- or two-way analysis of variance (ANOVA) with Bonferroni's posttest or Dunnett's multiple comparison tests, where appropriate. Microsoft Excel was used for *t* test and fold change analysis. Data of at least three ($n \geq 3$) experiments were evaluated to establish two-sided significance levels of independently reproduced determinations.

3. Results

3.1. Butyrate consumption by colon cells

Table 2 shows how primary nontransformed colon cells utilized butyrate, which was dissolved and diluted according

t2.1 Table 2
t2.2 Consumption of butyrate by human primary colon cells, determined as residual butyrate in the cell culture medium after 2, 4, 8 ($n=3$–5) and 12 h ($n=2$) incubation with butyrate

t2.3		mmol/L in the medium		Estimated cellular concentration (mmol/L)		Estimated uptake (μmol/l×10^6 cells)
t2.4		Mean	S.D.	Mean	S.D.	
t2.5	2 h	8.9	1.2	1.2	1.2	0.72
t2.6	4 h	9.0	1.1	1.5	0.5	0.9
t2.7	8 h	9.0	0.2	1.1	0.3	0.66
t2.8	12 h	10.7	0.4	0.5	0.1	0.3

t2.9 10.5±0.7 mM butyrate were detected as the starting concentration using gas chromatography.

to its molar mass (110.09 g/mol, sodium butyrate) to yield 10 mM in the culture medium. The estimated concentrations consumed by the cells were determined by analyzing the residual concentration of butyrate in the supernatants of treated cells.

As the starting concentration, 10.5 mM butyrate were detected with gas chromatography. This aberration from the calculated concentration can be due to technical conditions since the internal standard isocaproic acid was used as the basis of calculation.

After 2, 4 and 8 h, by calculating the difference between the measured starting and the residual butyrate concentration, on average, 1.2, 1.5 and 1.1 mM were consumed by the cells, whereas after 12 h, the concentration in the cell culture medium was lowered by approximately 0.5 mM. This was estimated to be the available amount of butyrate in the intracellular compartment. Thus, most of the added butyrate (86% after 4 h and 95% after 12 h) stayed detectable in the culture medium, pointing to a limited absorption process within 2–12 h ex vivo.

3.2. Viability, cell number and metabolic activity

Cell viability and cell number were determined by trypan blue exclusion after short-term treatments of single cells with butyrate. The treatment of primary colon cells for 0.5 to 4 h with the highest concentration of butyrate (50 mM) resulted in a number of biological effects. After 30 min, viability remained unchanged by the treatments, but cell recovery was below 100% for all data points, including the untreated control. After 1 or 2 h, the different concentrations again caused no impairment of the cells' viabilities, but the recovered numbers of cells decreased, mostly independent of increasing butyrate concentrations by almost 50%. After 4 h, cell viability was reduced in the three highest test concentrations (78.1%, 73.9% and 73.1%) in comparison to the baseline viability (90.4%). There was also a more pronounced reduction of 60% of cell number which, however, again was only marginally related to the butyrate concentrations (data not shown).

There were also different effects on metabolic activity by the butyrate treatment. After 2 and 4 h, the increase of the metabolic activity by butyrate (18.6% after 2 h and 31.5% after 4 h, 50 mM) may reflect trophic effects, which were lost and not any longer apparent for the extended treatment durations of 6 and 8 h (data not shown). In parallel studies (Sauer et al., in preparation), butyrate did not increase but rather reduced the metabolic activity more after 12 and 24 h at the same concentrations of ≥25–50 mM in cells from other donors.

When comparing the absolute levels of metabolic activity, the level increased from 2 to 4 h (3229±1455–3991±966 fluorescence units) and then was again reduced after 6 and 8 h (3609±1118 and 3113±1136 fluorescence units; data not shown).

3.3. Gene expression analysis

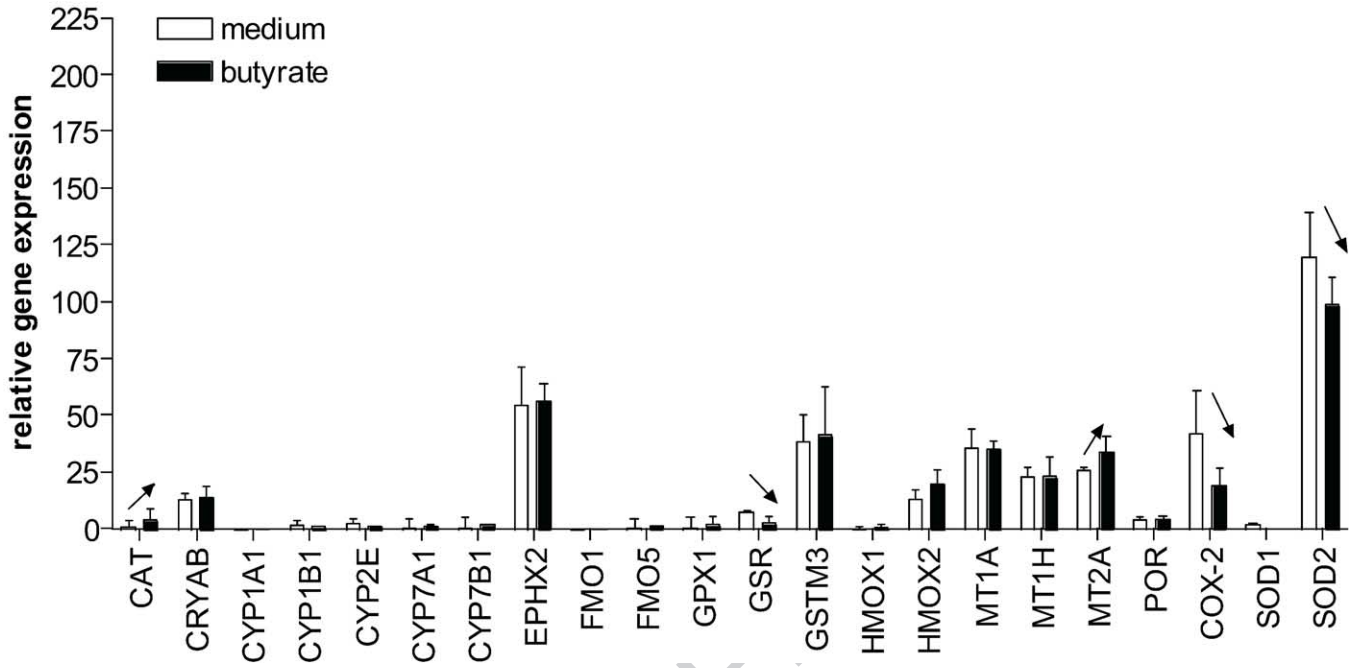
In analogy to previous studies [20], we incubated the intact epithelial stripes for 12 h (which was the maximal possible exposure time) with 10 mM butyrate (amount still within the nontoxic concentration range) to determine effects on patterns of gene expression using the HS12 cDNA macroarray (Superarray). Here, only results are reported on expression of genes related to oxidative and metabolic stress (Table 3). Further results of other gene groups will be described and discussed in another context elsewhere (Scharlau et al., in preparation), but are already posted on our Web site (www.uni-jena.de/biologie/ieu/et).

In the gene cluster of 22 oxidative and metabolic stress-associated genes, *hCAT* (2.9-fold) and *hMT2A* (1.3-fold; $P<.05$) were enhanced; *hGSR* (0.4-fold), *hPTGS2* (COX-2; 0.5-fold; $P<.001$) and *hSOD2* ($P<.05$) were lowered. As we have reported previously using the drug metabolism array (Superarray), a number of genes coding for cytochrome P450 enzymes were expressed only at very low levels and hardly altered by the butyrate treatment [20]. Similar results were obtained for the CYP450, spotted on the membrane used in this study (data not shown).

3.4. Confirmatory experiments with real time PCR

The reduction for *hCOX-2* and *hSOD2* mRNA and the induction of *hCAT* in primary colon cells after butyrate treatment (10 mM) was reassessed with real-time PCR. When investigating aliquots of the same RNA batches as those used in the array analysis ($n=3$), the directional changes of the gene expression levels were confirmed. Thus, expression levels for *hCAT* were increased with a fold change of 1.6±0.6, whilst expression levels of *hCOX-2* were reduced (fold change 0.7±0.2), in comparison to the medium control. Expression levels for *hSOD2* were also

t3.1 Table 3
t3.2 Modulation of gene expression in primary colon cells after treatment with 10 mM butyrate for 12 h. Mean±S.D. of the medium controls of three independently reproduced gene arrays are shown using RNA of three different donors (No. 1–3)



t3.4 Oxidative and metabolic stress	Expression level in the medium control		Fold change by treatment with butyrate		Analysis of significance	
t3.5	Mean	S.D.	Mean	S.D.	*t* test	2-way ANOVA
t3.6 CAT	4	9	2.9			
t3.7 GSR	24	4		0.4		
t3.8 MT2A	84	4	1.3		$P<.05$	
t3.9 COX-2	137	61		0.5		***
t3.10 SOD2	384	62		0.9		*

t3.11 For the butyrate treatment, fold change values are shown whereas a regulation is considered to be significant when ≤0.5 or ≥2.0, respectively. Statistical significance was checked using both an unpaired *t* test as well as a two-way ANOVA with Bonferoni's posttest, respectively.

422 reduced (fold change 0.7±0.4). The available RNA was not
Q7 423 sufficient to perform confirmatory analysis for GSR and
424 MT2A.

RNA from additional donors of primary colon cells 425
treated with butyrate under the same conditions was also 426
used to assess expression of *hCOX-2*, *hSOD2* and *hCAT*. 427

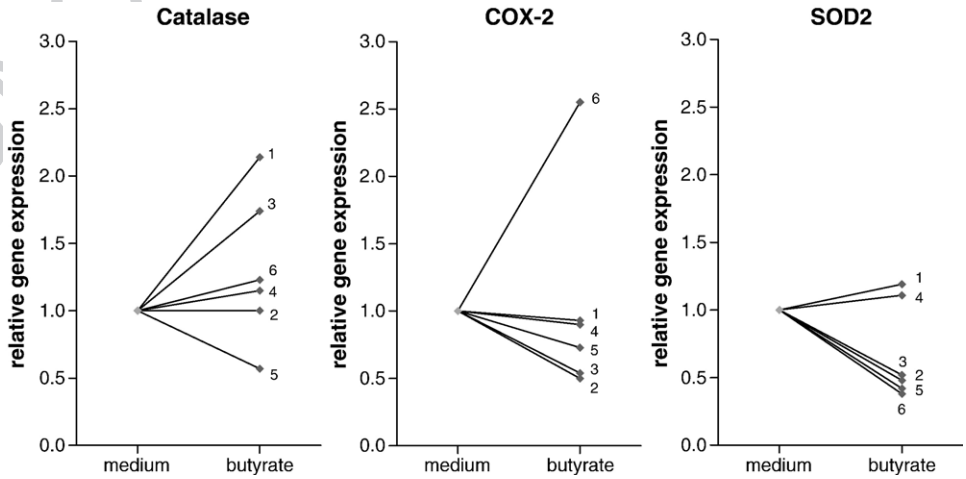


Fig. 1. Expression of *CAT*, *COX-2* and *SOD2* genes and variability of response with samples from different donors of primary colon cells after incubation with butyrate (12 h) analysed with real time PCR ($n=6$). Donors 1–3 also provided the RNA used in the gene expression analysis. When regarding results from all six donors, the changes mediated by butyrate treatment were nonsignificant using paired *t* test.

t4.1 Table 4
t4.2 GAPDH normalized expression levels (arbitrary units) calculated from the Ct values obtained in the real-time PCR experiments

t4.3 Donor	Reason for surgery	CAT			COX-2			SOD2		
		Expression level		fold change	Expression level		fold change	Expression level		Fold change
t4.4 t4.5		Medium	Butyrate		Medium	Butyrate		Medium	Butyrate	
t4.6 1	Anastomosenstenosis	0.014	0.029	2.14	0.330	0.308	0.93	0.250	0.297	1.19
t4.7 2	Adenoma	0.025	0.025	1.00	0.129	0.065	0.50	0.366	0.177	0.48
t4.8 3	Sigma carcinoma	0.012	0.021	1.74	0.297	0.159	0.54	0.467	0.241	0.52
t4.9 4	Sigma diverticulitis	0.074	0.085	1.15	0.225	0.203	0.90	0.203	0.225	1.11
t4.10 5	Sigma diverticulitis	0.088	0.051	0.57	0.125	0.092	0.73	0.210	0.088	0.42
t4.11 6	Sigma carcinoma	0.046	0.056	1.23	0.069	0.177	2.55	0.189	0.072	0.38

t4.12 Donors 1–3 were also used for the gene array analysis. The expression levels of catalase were lower than those for COX-2 and SOD2. Expression of all three genes was found to be highly variable between the different donors.

Fig. 1 shows that there is a high variability between the donors. With these samples (No. 4–6), the expression of *hCOX-2* was down-regulated in one of the additional donors, remained almost unchanged in the second and was distinctly up-regulated in the third of the new donors. For the *hCAT*, we also saw a variation. In two of the further donors, the expression was only slightly enhanced, whereas in the third new donor, it decreased. We found a similar pattern of variation for *hSOD2*. For this gene, the expression of one additional donor (as well as of one array-RNA donor) was slightly increased, whereas when using RNA of the two more donors, expression levels were reduced. Regarding the absolute values of the expression levels, there was also a strong variability between the different donors (see Table 4), but we were not able to attribute the variability to the type of disease. Moreover, the findings were not due to possible experimental artifacts either since both melting curve analysis and gel electrophoresis ascertained the specificity of the PCR products.

3.5. Measurement of catalase activity

After treatment of single cells for 30 min and 2 h with butyrate, we determined catalase activity in six different donors. Since the level of enzyme activity varied between the different individuals, we analysed changes of enzyme activity due to the treatment with butyrate as relative values after setting the corresponding medium controls to equal 100%.

After 30 min, we did not detect any change of catalase activity after butyrate treatment, whereas after 2 h, catalase activity was enhanced by about 65% subsequent to the butyrate treatment (Fig 2; $P=.0646$, unpaired t test).

We also treated cells isolated from three other donors for 4 h and 8 h. After 4 h, catalase activity also tended to be induced (18.4%), whilst after 8 h, the induction was less apparent (10.1%) (results not shown).

4. Discussion

Butyrate has been suggested to reduce cancer risks by acting as a cancer-suppressing type of agent [35,36]. If these effects also would also occur in vivo, it may be of exceptional protective activity under the "real-life situation," since in normal colon cells, it serves as a survival factor, and in transformed cell lines, it has been shown to inhibit the motility and to induce cell cycle arrest, differentiation and apoptosis [37,38]. Since humans steadily develop preneoplastic and neoplastic lesions with increasing age [39], a continuous exposure to butyrate could thus be meaningful in this context. In normal cells, which reflect more the types of cells available in a healthy, nondiseased colon mucosa, butyrate has trophic and growth-promoting effects and acts as a nutrient [40], enhances survival [38] and helps maintaining the integrity of the healthy colon mucosa [41,42]. Using normal primary human colon cells in culture is a unique in vitro possibility to study direct effects of butyrate in the actual target cells of colon carcinogenesis and to evaluate how the detected activities may be associated with potential mechanisms of chemoprevention. The approach, however, is relying on only a model system, and studies in humans will be necessary to determine more precisely the relevance of the findings for the in vivo situation.

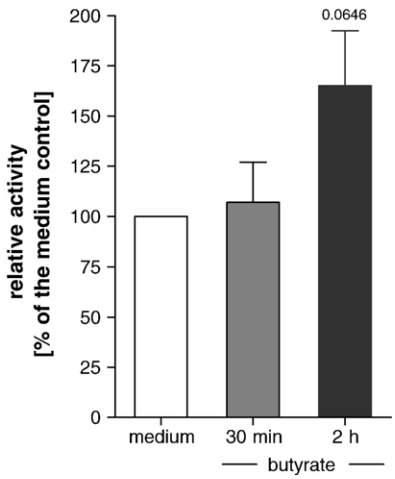


Fig. 2. Quantification of catalase activity after treating primary colon cells with butyrate (10 mM) for 30 min and 2 h. Cells treated with plain medium served as a control, which was set to equal 100% ($n=6$). Statistical evaluation with an unpaired t test (Welch's correction) revealed that the changes did not reach significance ($P=.0646$). The measured activity was based on the total protein concentration, as determined with the method of Bradford [32].

Here, it was shown that 1.5 mM of butyrate was taken up by primary cells treated with 10 mM butyrate after 4 h, whereas 0.5 mM were taken up after 12 h. These amounts represented only 14 and 5% of the exposure. To our knowledge, this is the first time butyrate consumption was directly measured in human colon cells in vitro. In rats, butyrate absorption increased linearly with luminal concentrations. It must be kept in mind, however, that higher butyrate concentrations may be needed in vivo than in vitro to achieve the same intracellular concentrations due to the presence of mucus or to differences in the absorptive surface of the cells in vivo [43]. Another study has shown that there is a regional variation of nutrient utilization in the colon using biopsy specimens [44]. In the colon, the absorbed butyrate is partly secreted out of the cell and reaches the blood stream [43], which may be an explanation for the increase of the detected butyrate amounts after 12 h in this study. Recently, we had shown that HT29 cells consumed 0.17 mM after 24-h treatment with 1 mM butyrate or 0.27 mM after treatment with 2 mM, equalling approximately 20% of the exposing dose. LT97 adenoma cells consumed 0.83- and 0.76-mM treatment with 1 or 2 mM butyrate for 24 h (80% and 75% of the original dose), respectively [29]. So far, we have not performed uptake studies with higher doses or shorter incubation times in the cell lines, but a comparison is currently being performed. Thus, in the half of the incubation time (12 vs. 24 h) primary colon cells consumed the double amount of butyrate (0.5 vs. 0.2–0.3 mM), which would speak for the trophic effects in primary cells, in comparison to HT29 cells, albeit culture media and exposure conditions were different. Different butyrate uptakes may be explained by the availability of the monocarboxylate transporter 1 (MCT1) which is necessary for butyrate uptake and is down-regulated from normal cells to malignant cells. Especially, normal cells require both the MCT1 transporter and butyrate for their homeostasis [45,46]. It also has to be considered that there is a time-dependant loss of cells, which means that the uptake per 1×10^6 cells must be regarded as an approximation.

Here, additional studies on the cytotoxic potential of a butyrate treatment in primary colon cells using metabolic activity, viability and cell number as parameters, were performed. The observed time-dependent effects indicated that individual primary cells in suspension culture remained viable for only relatively short periods of time (<<8 h). The loss of cells was time-related but was not enhanced with increasing concentrations of butyrate. After treating intact epithelial stripes, however, the viability of the subsequently isolated primary cells was retained for 12 h. Using these in vitro conditions, it was, moreover, possible to isolate sufficient intact RNA for further gene expression analysis. In the donors used for these experiments, butyrate did not impair the cells' metabolic activity but enhanced this parameter after short treatment durations (2 and 4 h) which again pointed to trophic effects.

The studies on modulation of gene expression by butyrate identified new target genes related to stress response in primary, nontransformed colon cells. The gene products are known to protect against factors of oxidative and metabolic stress. A confounding result was, however, that there was a high variability of gene induction by butyrate in cells from different donors. Thus, butyrate was strongly effective in cells of some donors, whereas cells of other donors did not respond. This finding necessitates further in-depth studies to elucidate the reasons for individuals being responders or nonresponders. In particular, for catalase, it is known that there is a common polymorphism in the promoter region of the *CAT* gene, which results in lower enzyme activity. It was also shown that the catalase activity is strongly affected by diet, especially by the consumption of fruits and vegetables, which deliver exogenous antioxidants [47]. Therefore, lifestyle factors of the donors of colon cells may be the reason for the variability of induction. The interindividual variability of gene expression levels is possibly due to unavoidable experimental differences encountered during the surgical isolation. However, the isolation procedure and the duration were always performed according to one standard protocol. In summary, it is clear from the present studies that butyrate does modulate gene expression in nontransformed primary human colon cells in vitro, as was shown by array analysis and real-time PCR.

Major findings were that *hCAT* and *hMT2A* were inducible in the primary colon cells. This suggests a better protection of butyrate-treated cells during situations of metabolic and oxidative stress. For example, the induction of catalase can protect the cells against H_2O_2, which is also produced endogenously [48]. A lower exposure of the cells to H_2O_2 (and, thus, to resulting reactive oxygen species) can protect cells from DNA damage, risk of mutations and possibly, initiation [49]. The effects by butyrate can be regarded to be chemoprotective for the untransformed cells, since exposure would be reduced. The induction of *MT2A,* as observed here, and of *GSTT2*, as observed previously [20], can both also protect from oxidative stress due to antioxidative capacities, particularly if the induction is present prior to oxidative stress [50]. For these genes, however, further confirmatory data is needed before coming to final conclusions.

This study also reports a reduced expression of *COX-2* in primary cells treated with butyrate, possibly resulting in anti-inflammatory mechanisms. The effect can be regarded to be protective [51], since the inhibition of chronic inflammatory processes might prevent enhanced proliferation in inflamed tissue [22]. An overexpression of *COX-2* has been reported in various types of tumors and some precancerous tissues. For instance, the inhibition of COX-2 activity was able to reduce growth of polyps in adenomatous polyposis coli knockout mice [52]. Cyclooxygenases are responsible for the metabolism of arachidonic acid into prostaglandins. There are two isoforms, the constitutively expressed COX-1 and the inducible COX-2, of which the

latter is implicated in tumorigenesis and cancer progression [53]. Altogether, the reduction of inflammatory processes may represent a feasible approach of chemoprevention in healthy cells. Of cause, it still remains to be demonstrated that similar effects can be measured in vivo.

In contrast, *SOD2* and *GSR* were reduced in primary cells. These genes code for two enzymes that are also important for the detoxification of products derived from oxidative stress, as are catalase and MT2A. Superoxide dismutase scavenges superoxide anions, which are reduced to H_2O_2 that is damaging. In following reactions, a glutathione peroxidase can cleave H_2O_2 to yield H_2O. For this reaction, glutathione is oxidised and can be reduced by the GSR. Since *SOD2* is expressed at a high level in the studied donors, the reduction might be not of such potent biological relevance. Moreover, our previous studies have shown that a preincubation of colon cells with butyrate could reduce the genotoxicity of H_2O_2 [18], possibly pointing to a more clear-cut functional consequence resulting from the induction of catalase and *MT2A* than by the reduction of *SOD2* and *GSR*.

In conclusion, even though only relative small quantities of butyrate were consumed by the primary colon epithelium, the intracellular concentrations were apparently sufficient to modulate gene expression. Our studies showed that physiological butyrate concentrations were not toxic to primary human colon cells. In particular, here, we present new insights into feasible approaches of chemoprevention in a nontransformed primary colon cell model since the enhancement of catalase, and potentially of other genes involved in the defense against reactive oxygen species, could protect cells from oxidative stress, whereas the repression of the COX-2 expression level could decrease inflammatory reactions posing a risk for the development of colon cancer.

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2.5 Publikation V: OVERVIEW OF EXPERIMENTAL DATA ON REDUCTION OF COLORECTAL CANCER RISK BY INULIN-TYPE FRUCTANS.

Beatrice L. Pool-Zobel und **Julia Sauer**. Eingereicht beim *Journal of Nutrition (Supplement on invited conference presentations)* November 2006

Die Entstehung von Dickdarmkrebs steht in enger Beziehung zur Ernährungsweise. Zahlreiche *in vitro* und *in vivo* Studien konnten zeigen, dass Fruktooligosaccharide über die Bildung von kurzkettigen Fettsäuren einen protektiven Einfluss ausüben können. Butyrat führt zu Apoptose in Krebszellen, reduziert die Metastasierung und schützt durch Induktion von Entgiftungsenzymen vor genotoxischen Substanzen. Für Fermentationsüberstände aus Inulin konnte auch eine Hemmung des Wachstums von Kolonadenom- und Adenokarzinomzellen gezeigt werden. In primären Kolonzellen wurde durch Fermentationsprodukte die Expression von Katalase induziert. In Tierstudien konnte durch Fruktooligosaccharide eine AOM-induzierte Kolonkarzinogenese verhindert werden. Weitere *in vivo* Tierstudien beschreiben eine Verringerung chemisch induzierter präneoplastischer Läsionen nach Fütterung von Fruktanen. In einer Humanstudie (SYNCAN-Projekt) wurde der Einfluss einer Fruktan-Intervention auf die Reduktion des Kolonkrebsrisikos untersucht. Es gibt zahlreiche experimentelle Beweise, dass Inulin das Kolonkrebsrisiko modulieren kann. Die zugrunde liegenden Mechanismen könnten die Verringerung der Exposition gegenüber Risikofaktoren sowie wachstumshemmende Effekte auf Tumorzellen beinhalten.

Eigenanteil:

- Isolierung und Aufarbeitung der Kolongewebeproben; Inkubation der Zellen
- Messung der Vitalität und metabolischen Aktivität
- RNA-Isolation und Durchführung der real-time PCR und Messung der Katalase-Aktivität
- Auswertung, Interpretation und Darstellung der Ergebnisse
- Zusammenstellung der aktuellen Datenlage der Literatur; Verfassung des Manuskriptes

Overview of experimental data on reduction of colorectal cancer risk by inulin-type fructans[1]

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Running title: Reduction of colon cancer risk by inulin

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Abstract

Colorectal cancer is related to diet, lifestyle, physical inactivity and obesity. The responsible carcinogens cause mutations or enhance cell growth. Inulin-type fructans may counteract the effects via their gut flora-mediated fermentation products *in vitro* and *in vivo*. Important products formed by fermentation of inulin with human gut flora are short chain fatty acids. Of these, butyrate and propionate inhibit growth of colon tumor cells and histone deacetylases. Butyrate also causes apoptosis, reduces metastasis in colon cell lines, and protects from genotoxic carcinogens by enhancing expression of enzymes involved in detoxification. Fermentation supernatants of inulin have similar growth-inhibitory effects on colon adenoma and carcinoma cells and induce histone hyperacetylation by inhibiting histone deacetylases. In animal models inulin-type fructans prevent and retard colorectal carcinogenesis. Several studies reported the reduction of chemically induced pre-neoplastic lesions or tumors in the colon of rodents treated with inulin-type fructans. The human intervention study (SYNCAN project) aimed to attenuate the experimental evidence for risk reduction by inulin-type fructans in humans. One group of polypectomized people at high risk for colon cancer and another of colon cancer volunteers after curative resection were given a synbiotic preparation. There were clear functional effects of the synbiotic since numerous different cancer risk markers were favourably altered. In conclusion, there is considerable experimental evidence that inulin modulates parameters of colon cancer risks in human colon cells, in animals and in a human intervention trial. The involved mechanisms possibly include reduction of exposure to risk factors and suppression of tumor cell survival.

KEY WORDS: • inulin • colon cells • colon cancer

Abbreviations used: ACF, aberrant crypt foci; AOM, azoxymethane; APC, adenomatous poliposis coli; GSTs, glutathione S-transferases; H_2O_2, hydrogen peroxide; SCFA, short chain fatty acids; SFS, Synergy1 fermentation supernatant; TEER, transepithelial electrical resistance.

Introduction

The evolution of CRC is a complicated multi-step process involving specific molecular genetic alterations in tumor suppressor genes, protooncogenes and genes encoding proteins for DNA repair (1). Some of these genetic lesions may be inherited and are the most decisive type of susceptility factors (2). The alterations can also be caused during the lifetime of an individual by exogenous and endogenous chemicals with genotoxic potential (3). They have their source in the general environment, in nutrition, and subsequently in various processes of metabolically endogenous conversion (4). This leads to a considerable burden of toxic and genotoxic factors in the gut lumen. Fecal samples for instance have been shown to contain

- Bile acids, amines, sulphates, and bacterial toxins (5)
- Additional products of bacterial biotransformation (6)
- Non-digested food residues and excretable metabolites and
- Genotoxic compounds (7).

Collectively, the exposure to such substances may initiate the process of colorectal carcinogenesis or enhance its progression.

Protective factors from nutrition, such as foods containing inulin-type fructans, may lead to other fecal compounds which are more related to cancer prevention, such as the short chain fatty acids (SCFA), of which butyrate has been shown to have numerous biological effects. This product of gut flora-mediated fermentation induces apoptosis of tumor cells (8) and protects cells from genotoxic insult by elevating phase II detoxification (9). The SYNCAN-project has investigated some of these mechanisms in more detail as is outlined below.

In vitro studies

In a series of experiments performed *in vitro* with cultured cell lines we first investigated the composition and the effects of a fermentation supernatant obtained after anaerobic incubation of Synergy1 (a prebiotic mixture of oligofructose and inulin) with samples of feces-derived

human gut flora (10). A subgroup of fermentation samples representing different regions of the large intestine, were prepared as described (11). As shown in Figure 1, the butyrate concentration was markedly increased in vessel 3 which mimics the type of fermentation occurring in the distal colon. It is also apparent from Figure 1 that especially the fermentation products formed in vessel 3 impaired the survival of tumor cells and were the most efficient inhibitors of cell proliferation. At the same time, markers indicative of the intestinal barrier function were modulated since TEER was lowered while mannitol flux increased. There was also a trend of fermentation products to inhibit invasion (10). Together, the information obtained thus far strongly indicates that fermentation supernatants derived from Synergy1 impair growth, survival and progression of human tumor cell lines, all mechanisms which are associated with suppressing activity and secondary cancer prevention (12,13).

An important mechanism by which butyrate causes biological effects in colon carcinoma cells has been proposed to be the hyperacetylation of histones by inhibiting histone deacetylases (14). Histone acetylation influences gene transcription by loosening histone-DNA contacts, thus making the DNA accessible for transcription factors. Imbalance in histone acetylation can lead to transcriptional dysregulation and silencing of genes that are involved in control of cell-cycle progression, differentiation, apoptosis and cancer development. Recent studies have shown that butyrate and propionate enhanced histone acetylation in HT29 colon adenocarcinoma cells. Together, these SCFA could possibly mediate important processes related to an altered transcriptional gene activation and thus contribute to biological effects possibly related to cancer progression or prevention (15).

Next, we were interested in effects of inulin fermentation products in primary cells. In particular, it was of interest to assess activities which would prohibit the formation of initiated cells, or in other words, prevent the onset of carcinogenesis. For this, we continued our efforts of the last decades (16-18) to refine methods for obtaining primary, non-transformed healthy colon cells and keeping them alive *in vitro* for a reasonable period of time. Our newest

development now is to use surgical samples from which we isolate the epithelial layer and prepare either epithelial tissue pieces or isolate intact cells (19). These primary cells, as isolated cells in suspension culture, retain their viability for approximately one hour. However, if intact tissue stripes are incubated *in vitro* and then individual cells are isolated, sufficiently viable cells with > 50-70 % survival can still be recovered after 12 hours of *in vitro* culture. Thus, it is now also possible to perform *in vitro*, cell-based studies for up to 12 hours (20,21). Using these *in vitro* cultivation techniques a first aim was to assess survival of primary colon cells exposed to the Synergy1 fermentation supernatants (SFS) and to corresponding controls. Figure 2 shows effects of these samples on mitochondrial metabolic activity. There is a marked increase of metabolic activity in primary colon cells treated with SFS. This increase indicates that the fermentation compounds are utilized by the colon cells, an effect which possibly reflects trophic effects. The enhanced metabolism, however, is not caused in a similar manner by butyrate which has been reported to be a survival factor for primary colon cells before (22). Figure 2 also shows that the effects are also not due to a mixture of SCFA composed to mimic the SFS. Instead, it rather more seems that the feces supernatant is supplying survival factors that “last” only for 12 hours, but not for 24 hours. Thus, the SFS seems to be more potent in increasing survival of primary colon cells on account of as yet unknown compounds of fecal origin but not on account of the SCFA. It will be of interest to identify and characterize those fecal survival factors more in detail in the future.

The studies also provide the experimental basis for determining various additional biological effects caused by SFS in primary human colon cells. One of the first applications was to study altered patterns of gene expression caused by SFS, SCFA and the corresponding controls. In particular, we have investigated expression of genes related to drug metabolism (18), stress response (21), proliferation and apoptosis (Scharlau et al, in preparation). The study of GSTs was of particular interest, since it seems likely that an induced expression of GSTs will result

in the protection of cells from genotoxic insult by specific chemicals (23). Especially since these particular enzymes are more involved in deactivation rather than in activation and since they are inducible (23,24). However, experimental verification of the hypothesis is needed. Butyrate has been shown to induce several types of GSTs in primary human colon cells (18). Recent studies have now shown that butyrate is also able to enhance expression of catalase, but inhibits cyclooxygenase 2 (COX-2) and superoxid dismutase 2 (SOD2) (21). Figure 3 shows that there is an induction of catalase, determined both on mRNA level using real-time PCR and on enzyme activity level. This increased catalase activity could result in a reduced genotoxicity by its substrate hydrogen peroxide (H_2O_2). In rat colon cells H_2O_2 is less genotoxic (see Figure 4) in butyrate pretreated cells than in the controls (25). It will now be of interest to determine if a butyrate pretreatment of human colon cells also results in a lower genotoxicity of H_2O_2 and how SFS are able to elicit similar effects.

In vivo studies

A number of studies report the effects of inulin-type fructans on chemically induced pre-neoplastic lesions (ACF) or tumors in the colon of rats and mice (26). Inulin-type fructans reduced tumor incidence in APC^{min} mice and reduced growth and metastasising properties of implanted tumor cells in mice. The most pronounced effects were reported for inulin-type fructans (designed for favorable fermentation in the colon lumen) and especially longer-chain inulin components (optimal effectiveness at 10% w/w in diet), animals fed a high-fat Western style diet, intervention together with probiotic bacteria (synbiotic preparations) and intervention throughout the whole carcinogenesis process (26). The effects have been reported to be associated with gut flora-mediated fermentation and production of butyrate. The results are meaningful according to the PASSCLAIM evaluation (24) since aberrant crypt foci are a valuable biomarker in rodents, providing a quantitative assessment of the development of colon cancer. The presence of adenomas and adenocarcinomas, their size and multiplicity are

directly linked to cancer, and the final resulting mortality is a hard endpoint resulting from tumor progression. In conclusion, the studies on chemically induced pre-neoplasia and tumors in the colon of rats point to a clear-cut non-toxic effect of inulin-type fructans leading to a marked reduction of colon cancer incidence in animals exposed to the experimental colon carcinogens.

As a part of the SYNCAN-study this animal model was used to compare the efficacies of intervention with Synergy1 (prebiotic), bifidobacteria lactis Bb12 + lactobacillus rhamnosus GG (probiotics) and the combination of both (synbiotic). Rats treated with Synergy1 as prebiotic or synbiotic had a lower number of carcinogen-induced tumors, both adenomas and cancers (27). The model was also used to validate the biomarker fecal water genotoxicity. For this, feces were obtained from the AOM-treated rats at 2, 4 and 8 months after the beginning of the study. Fecal waters were prepared and analysed for DNA damaging potential using the single cell microgelelectrophoresis, “comet” assay (28). This study found a clear cut reduction of genotoxicities of fecal waters from prebiotic-treated rats after 4 and 8 months (see Figure 5). Fecal water genotoxicity was also decreased in the synbiotic group but not in the group receiving only probiotics. Finally, this biomarker is of interest and the PASSCLAIM group concludes that cytotoxicity and particularly genotoxicity of fecal water have a good mechanistic link with colon carcinogenesis and hence provide potentially valuable, non-invasive methods for assessing colorectal cancer risk in human subjects (24). However, there is a need for more extensive validation of these endpoints.

In the human intervention study the synbiotic treatment resulted in a significant reduction of the DNA damaging capacity of fecal water in polyp patients at the end of the intervention period but not in the cancer patients as shown in Figure 6 (29). Another human study (30) detected a decline of toxic bile acids in fecal samples after 3 months intervention with short-chain oligofructoses, a finding which confirms some of our observations. Another related biomarker of interest measured in the human SYNCAN trial was to investigate the changes of

DNA damage in colon cells of patients receiving the synbiotic intervention. Colonic biopsies were available from the same groups of patients from which fecal water had been obtained. The differences in the extents of DNA damage for polyp patients and for cancer patients are shown in Figure 7. Again the polyp patients seem to profit more from the intervention since there was a clear reduction of DNA damage after synbiotic treatment but not after placebo administration. According to the PASSCLAIM diet-related cancer group, the determination of DNA damage provides a useful tool to investigate the effects of diet in different human tissues which are relevant for diet-associated tumor occurrence (24). The modulation of DNA damage reasonably well reflects also the modulated exposure to genotoxic compounds that cause the damage. Therefore, the reduction of DNA damage will indicate a reduced exposure which in turn is related to a decrease of risk. The results may be more meaningful than those of the fecal water genotoxicity, and together with the other data from this SYNCAN trial, they do indicate that the intervention is reducing exposure to genotoxic compounds in the gut.

Conclusions

In conclusion, there are now data available that *in vitro* fermentation products of Synergy1, one of the most effective inulin-type fructans, modulate parameters related to CRC initiation and progression in a favorable/beneficial way. In animal experiments *in vivo* tumors are inhibited experiments and fecal water genotoxicity is reduced after intervention. In humans, fecal water genotoxicity is also reduced and moreover, damage in colon cells is inhibited.

Thus for the time being we may conclude „SYNCAN" meaning that **SYN**biotic-intervention **CAN** reduce exposure which in turn is related to a decrease of colon cancer risks. In rats numerous studies show that it prevents from chemical induced colon tumors. Whether it can prevent colorectal cancer in humans will now need long term prospective studies with cancer development as an endpoint.

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LEGENDS TO THE FIGURES

FIGURE 1: Concentration of the three main SCFA in the fermentation sample of Synergy1 in three different vessels of an *in vitro* fermentation system and the effects of the supernatants from vessel 2 and 3 on human tumor cells. Results have been published in (31).

FIGURE 2: Metabolic activity of primary human colon cells after incubation with a Synergy1 fermentation supernatant, a butyrate control, the feces control and a mixture of short chain fatty acids mimicking the concentrations in the fermentation supernatant for 4 to 24 hours. Results are from Sauer et al., *under current revision* (2006).

FIGURE 3: Modulation of catalase expression in primary human colon cells on mRNA level using real-time PCR (left side) and on protein level measuring enzyme activity (right side).

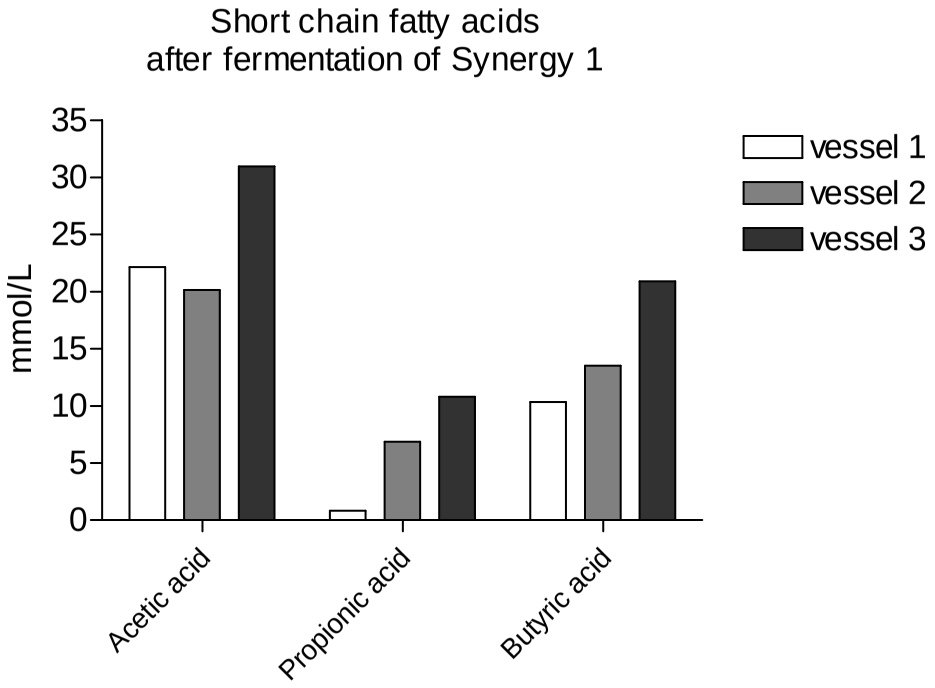
FIGURE 4: Effects of a butyrate pre-incubation for 15 min on H_2O_2-induced DNA damage detected with the Comet Assay in colonic epithelial cells (Adapted from Abrahamse et al.(25)).

FIGURE 5: Reduction of fecal water genotoxicity in AOM-treated rats after intervention with synbiotics (Klinder et al., (28)).

FIGURE 6: Reduction of fecal water genotoxicity in polyp patients after a 12 week intervention with synbiotics (Rafter et al, AJCN, *accepted* 2006). Fecal samples were obtained before, during and after the intervention. The study included polypectomised and colon cancer patients.

FIGURE 7: In the 12 week randomized, double blind, placebo-controlled trial of a synbiotic food composed of Synergy1 and the probiotics LGG and BB12 colorectal biopsies were taken before and after the intervention from colon cancer patients and polypectomised patients. DNA damage was measured in colon cells of the biopsies after intervention with synbiotics (Rafter et al, AJCN, *accepted* 2006).

FIGURE 1


Short chain fatty acids
after fermentation of Synergy 1
mmol/L
35
30
25
20
15
10
5
0
Acetic acid
Propionic acid
Butyric acid
vessel 1
vessel 2
vessel 3

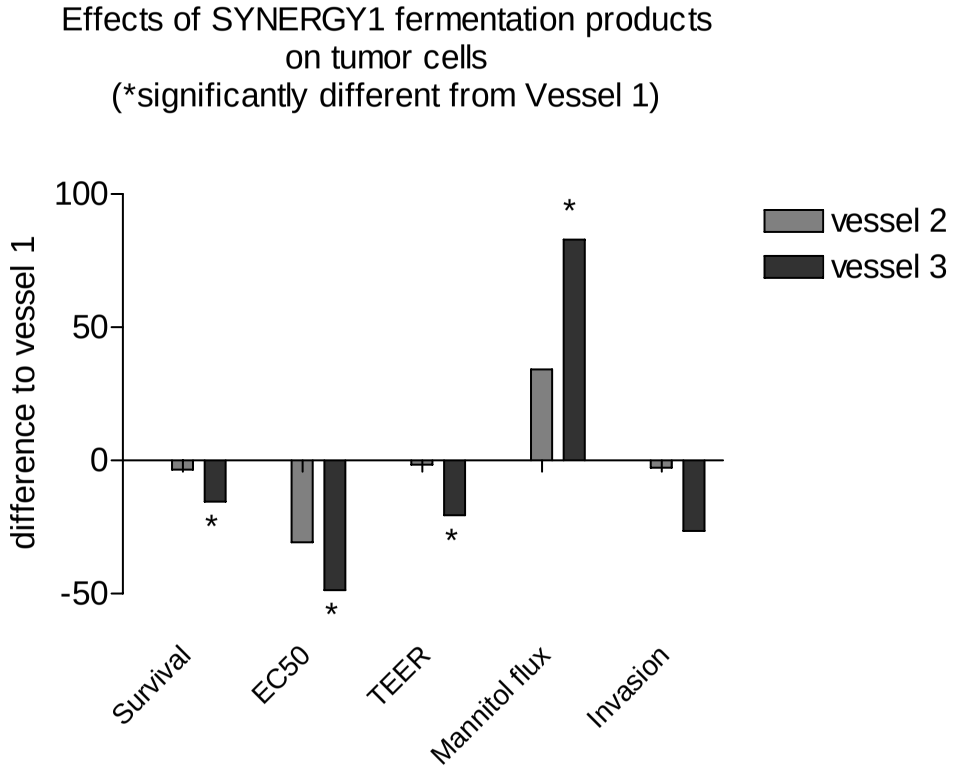

Effects of SYNERGY1 fermentation products
on tumor cells
(*significantly different from Vessel 1)
difference to vessel 1
100
50
0
-50
*
*
*
*
Survival
EC50
TEER
Mannitol flux
Invasion
vessel 2
vessel 3

FIGURE 2

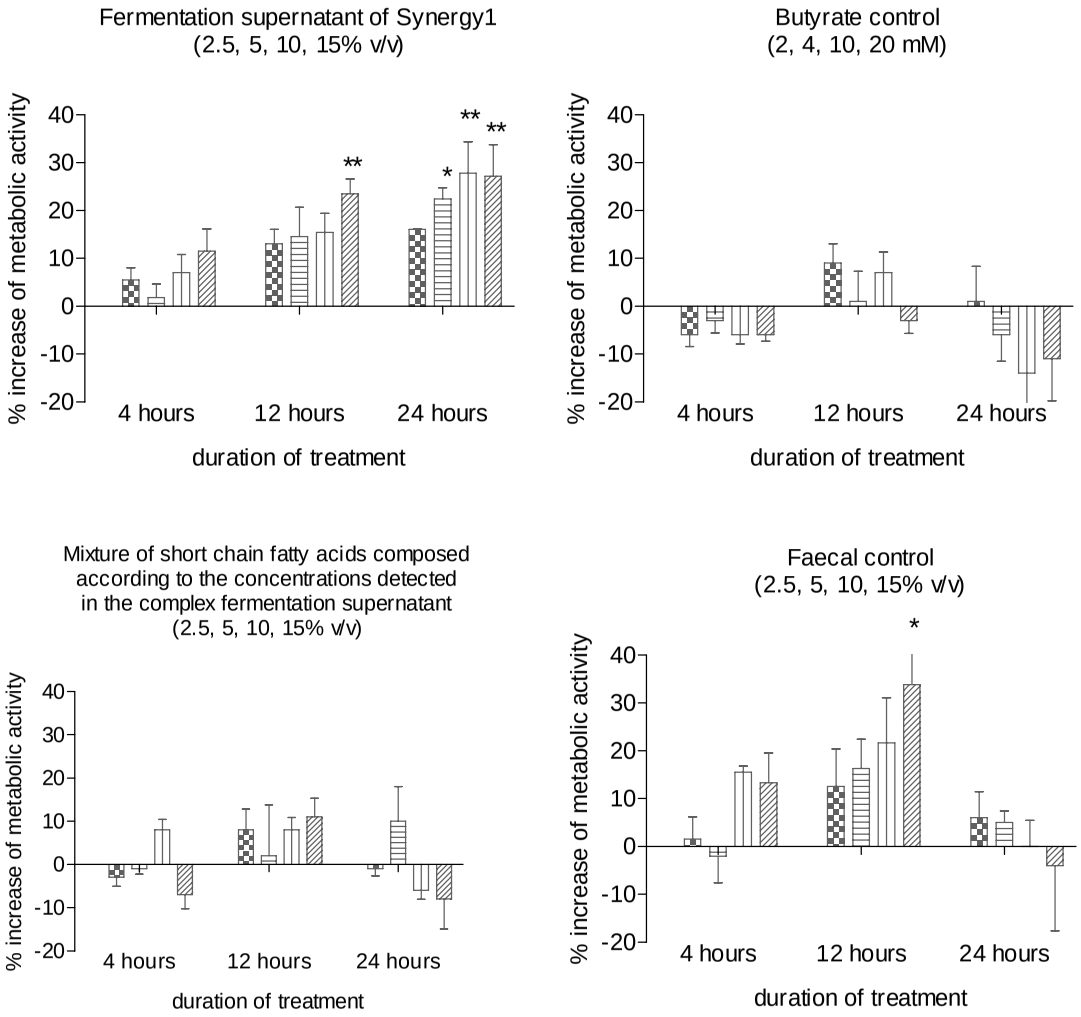
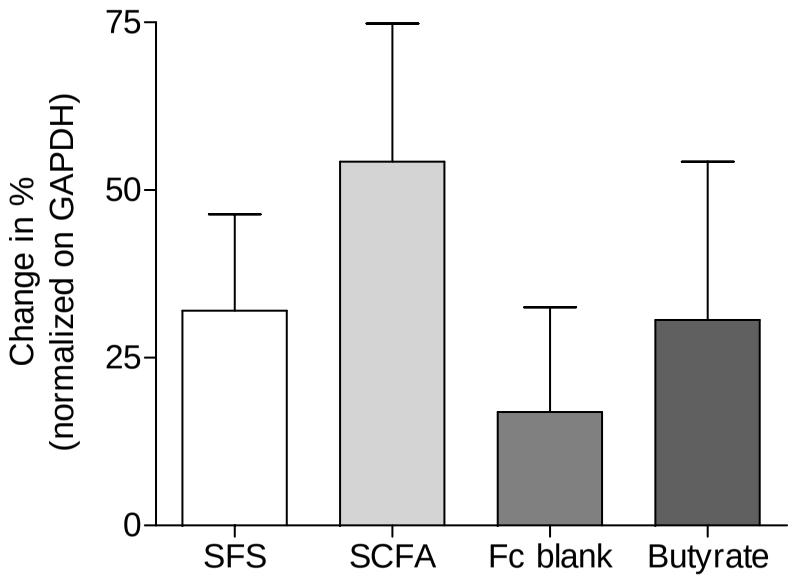

Fermentation supernatant of Synergy1
(2.5, 5, 10, 15% v/v)
% increase of metabolic activity
40
30
20
10
0
-10
-20
**
*
**
**
4 hours
12 hours
24 hours
duration of treatment
Butyrate control
(2, 4, 10, 20 mM)
% increase of metabolic activity
4 hours
12 hours
24 hours
duration of treatment
Mixture of short chain fatty acids composed
according to the concentrations detected
in the complex fermentation supernatant
(2.5, 5, 10, 15% v/v)
% increase of metabolic activity
4 hours
12 hours
24 hours
duration of treatment
Faecal control
(2.5, 5, 10, 15% v/v)
*
% increase of metabolic activity
4 hours
12 hours
24 hours
duration of treatment

FIGURE 3


Change in %
(normalized on GAPDH)
75
50
25
0
SFS
SCFA
Fc blank
Butyrate

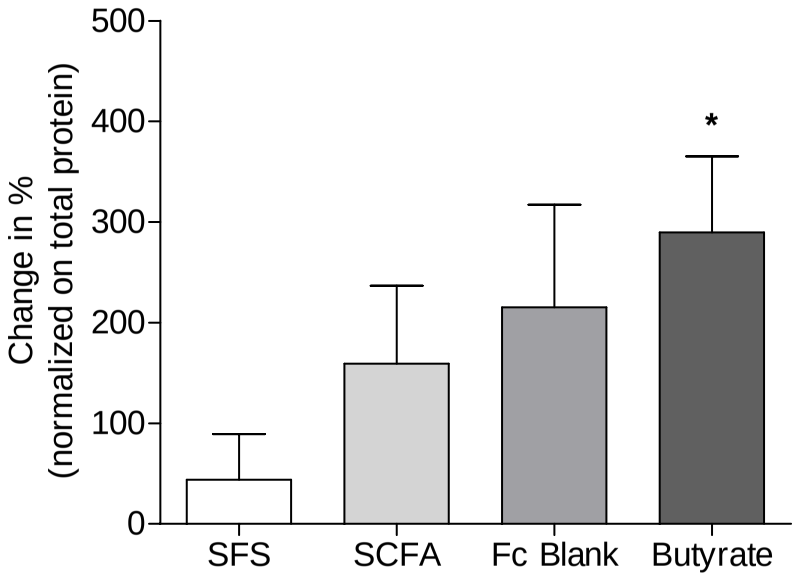

Change in %
(normalized on total protein)
500
400
300
200
100
0
*
SFS
SCFA
Fc Blank
Butyrate

FIGURE 4

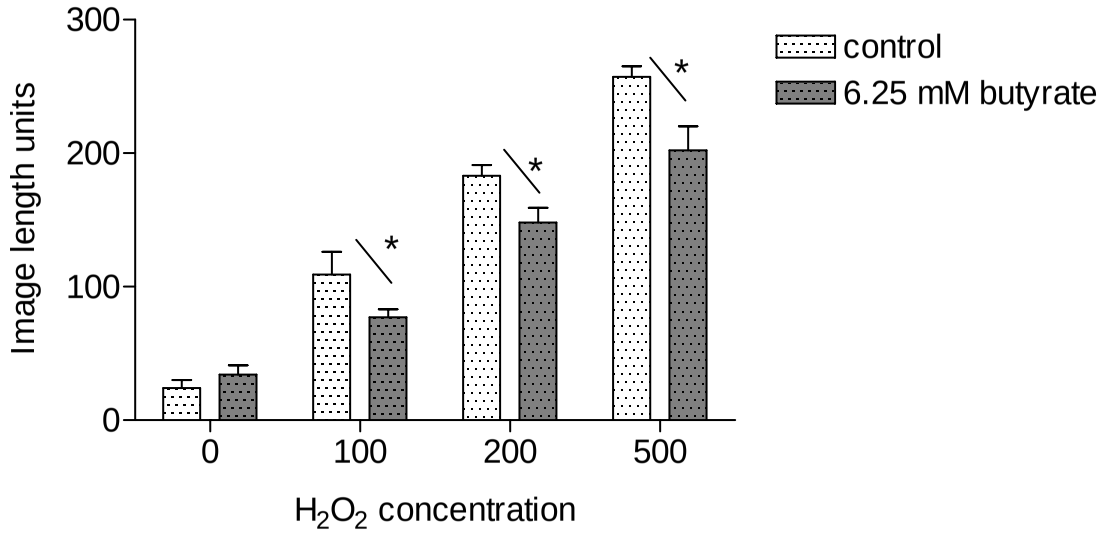

300
200
100
0
Image length units
0
100
200
500
H_2O_2 concentration
control
6.25 mM butyrate
*
*
*

FIGURE 5

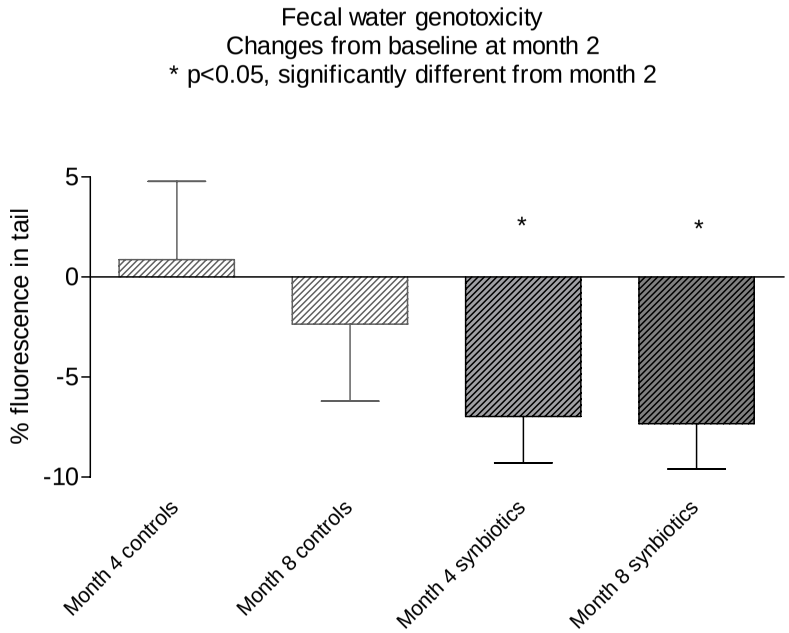

Fecal water genotoxicity
Changes from baseline at month 2
* p<0.05, significantly different from month 2
% fluorescence in tail
5
0
-5
-10
*
*
Month 4 controls
Month 8 controls
Month 4 synbiotics
Month 8 synbiotics

FIGURE 6

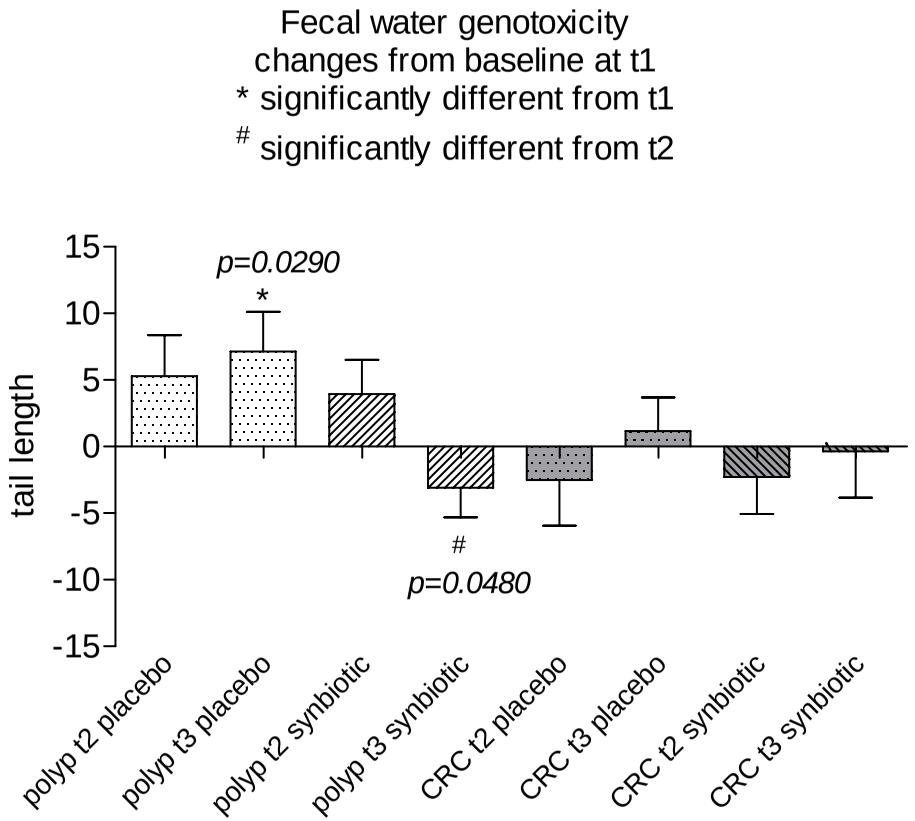
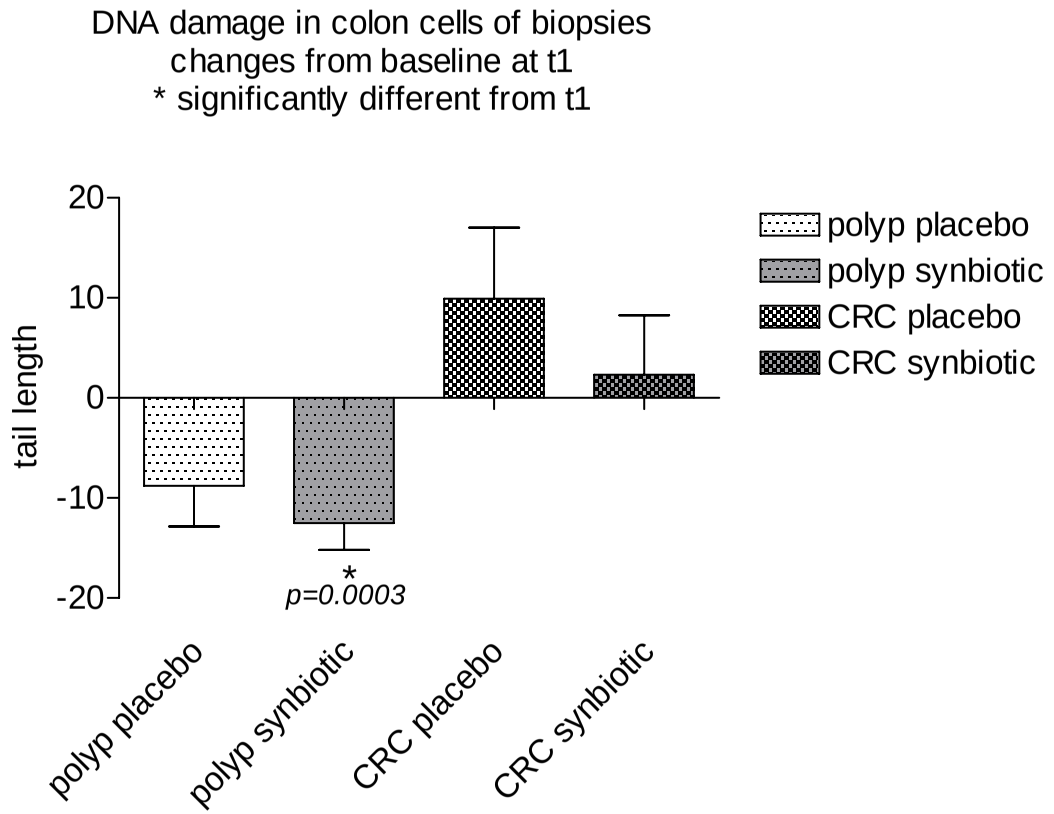

Fecal water genotoxicity
changes from baseline at t1
* significantly different from t1
# significantly different from t2
tail length
15
10
5
0
-5
-10
-15
p=0.0290
*
#
p=0.0480
polyp t2 placebo
polyp t3 placebo
polyp t2 synbiotic
polyp t3 synbiotic
CRC t2 placebo
CRC t3 placebo
CRC t2 synbiotic
CRC t3 synbiotic

FIGURE 7


DNA damage in colon cells of biopsies
changes from baseline at t1
* significantly different from t1
tail length
20
10
0
-10
-20
*
p=0.0003
polyp placebo
polyp synbiotic
CRC placebo
CRC synbiotic
polyp placebo
polyp synbiotic
CRC placebo
CRC synbiotic

2.6 Publikation VI: BUTYRATE DIFFERENTIALLY MODULATES SELECTED DETOXIFYING ENZYMES IN PRIMARY CELLS DERIVED FROM NORMAL AND ADJACENT TUMOR TISSUE.

Julia Sauer, Konrad K. Richter, Karl Otto Greulich, Brigitte Altenberg and Beatrice L. Pool-Zobel. Manuskript in Vorbereitung für *Carcinogenesis* 2007.

Für Butyrat wurden gegensätzliche Effekte auf normale Kolonzellen und Tumorzellen beschrieben. In nicht-transformierten Zellen wird Butyrat als Energiequelle gebraucht, während es in Tumorzellen das Wachstum hemmt und Apoptose induziert. In beiden Zelltypen kann die Genexpression durch Butyrat moduliert werden, wobei eine Steigerung der Entgiftungskapazität in normalen Zellen besonders wünschenswert ist. Im vorliegenden Manuskript wurden die basale Genexpression sowie der Einfluss von Butyrat auf normales und angrenzendes Tumorgewebe ein und desselben Spenders untersucht. Durch die parallele Behandlung beider Zelltypen eines Individuums kann die Butyratwirkung in Zellen unterschiedlichen Transformierungsgrades charakterisiert werden. Mit diesem Ansatz können weitere Gene identifiziert werden, die eine Rolle in der Kolonkarzinogenese spielen sowie interessante Targets für Mechanismen der ernährungsbedingten Primärprävention darstellen.

Eigenanteil:

- Isolierung und Aufarbeitung des normalen Kolongewebes sowie des Tumorgewebes; Durchführung der Inkubationen
- Messung der Vitalität
- RNA-Isolation und Durchführung der Gen-Arrays
- Durchführung der real-time PCR
- Auswertung, Interpretation und Darstellung der Ergebnisse
- Verfassung des Manuskriptes

Butyrate differentially modulates selected detoxifying enzymes in primary cells derived from normal and adjacent tumor tissue

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Running Title: Gene modulation by butyrate in normal and tumor colon cells

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Abstract

Introduction: Colorectal tumors are a common malignancy in countries with Western style diets. The consumption and gut-flora mediated fermentation of dietary fibre may protect from the development of colon cancers by the production of short chain fatty acids. For butyrate, contrasting effects are described for normal colonocytes and tumor cell lines. In normal cells, butyrate acts as a nutrient and modulates gene expression. In tumor derived cell lines butyrate inhibits proliferation, induces apoptosis and has different patterns of modulating gene expression. **Material and Methods:** Cells were isolated from primary non-transformed colon cells and adjacent tumor tissues co-removed during coloresection. Viability was assessed as a routine parameter for a successful cell preparation. RNA was directly isolated and used for gene arrays to compare basal gene expression levels of normal and tumor tissue of the same donor. The results were compared to expression data of selected genes using the dbEST database provided by the NIH. Further tissues were treated for 12 h with butyrate. RNA was isolated and real-time PCR was performed for GSTP1, GSTM2, COX-2 and CAT to measure distinct effects of butyrate in the two different cell types. **Results:** Viability did not differ between normal and tumor cells. The gene array analysis revealed that gene expression was similar for many of the pathway-specific genes but there were also several differentially expressed genes as shown by datamining. Butyrate modulated GSTP1 and GSTM2, COX and catalase with large interindividual variations of response between different donors and tissues. **Conclusions:** Using gene arrays and datamining, it was possible to identify genes differently expressed in normal and tumor cells from the same donor. After treating both cells types with butyrate the effects of the fermentation product in cells of different stages of carcinogenesis could be characterized. With this approach, genes can be identified which can be considered as targets in human colon carcinogenesis as well as genes which may represent targets in nutritional chemoprevention.

Keywords: butyrate, gene modulation, datamining, chemoprevention, primary normal and tumor cells

Introduction

Butyrate is a short chain fatty acid which is formed in millimolar concentrations during gut fermentation of dietary fibre and is known to have several colon chemopreventive effects [1]. *In vivo* it was shown that a high consumption of dietary fibres correlates with higher luminal butyrate concentrations [2]. Until now, there are epidemiological follow-up studies in humans analysing the intake of dietary fibre over years and recording the incidence of colon adenoma or carcinomas but are often confounded by unknown factors [3;4]. In animal models, higher luminal butyrate concentrations are inversely correlated with tumor size [5]. Moreover, in rat feeding studies aberrant crypt foci were reduced by the dietary fibre inulin after AOM application [6;7]. Inulin is fermented by the human gut flora to yield butyrate which is known to have several chemopreventive properties [8-10]. So far, there is a plenty of information about the effects of butyrate in different cell types of human or animal origin which can be classified into blocking and suppressing agent activities [11]. It is well documented that butyrate inhibits tumor cell growth and induces apoptosis and differentiation *in vitro* [12]. Contrarily, it promotes normal colon cells by which it is used as an energy fuel [13]. This phenomenon is often described as the so called "butyrate-paradox"[14]. A lot of *in vitro* studies usually used transformed cell models to study the effect of butyrate which give only limited answers for effects on normal, non-transformed human colon cells [15;16] but there is only little information on effects in primary human colon cells.

Moreover, paradoxical effects of butyrate on proliferation and differentiation has been described in freshly isolated colon cells compared to colon cancer cell lines which can be caused by the isolation process and the adaptation to *in vitro* conditions, respectively [17]. In the present study, a new approach to assess the chemoprotective potential of butyrate both in normal and in tumor cells is introduced. The rationale of the approach is that humans bear preneoplastic lesions (every 3rd to 2nd person at the age >60-70) next to normal colon cells [18;19], but are continuously producing butyrate (and other fermentation products) from

dietary fibre. It is therefore important from a nutritional point of view to characterize more in depth how fermentation products affect non-transformed and transformed cells in the human gut.

Several studies have shown that butyrate may influence the expression of detoxification enzymes. An enhancement of GSTs is described in human adenocarcinoma cell lines [16;20;21]. If the induction of detoxification enzymes also occurs in normal, non-transformed colon cells they may be better protected against colon cancer risk factors [22;23]. In general, an induction of the toxicological defence system may strengthen the cells' ability to detoxify colon cancer risk factors like products from lipid peroxidation and reactive oxygen species [16;24].

The basal gene expression and modulation of detoxification enzymes (e.g. GSTs) or of genes belonging to oxidative and metabolic stress (e.g. catalase, COX-2, SOD, GSR, GPx) by fermentation products is supposed to be different in normal and tumor cells [21]. Basal gene expression and the modulation of gene expression by nutritional compounds may also vary strongly between cells of different donors due to possible genetic polymorphisms which may be responsible for responders and non-responders [25].

In the work presented here, we directly compare cell viabilities, basal gene expression and a modulated state of gene expression by butyrate of selected genes in both freshly isolated normal and tumor cells derived from the same patient during surgery to estimate the individual responses of cells to a butyrate treatment in two different cell types. The results will provide new insights into distinct effects of butyrate on cells of different transformation stages from the same donor of which normal colon cells also represent the actual target cells of colon cancer development [26].

Materials and Methods

Isolation of primary human epithelium

Primary human normal and tumorous colon tissues were obtained from tissue specimens of the same donor during surgery of colorectal tumors from patients who had given their informed consent. The normal tissues were co-removed during the surgery due to medical indications and were taken as far as possible from the disease sites and by and large were considered to represent non-affected tissues. The university ethics committee approved the study. Mean age (±SD) of the donors of colon cells was xx±xx years, xx of the donors were male, xx were female. Both tissues were stored in HBSS (Hank's balanced salt solution; 8.0 g/l NaCl; 0.4 g/l KCl; 0.06 g/l $Na_2HPO_4 \times 2\ H_2O$; 0.06 g/l K_2HPO_4; 1 g/l glucose; 0.35 g/l $NaHCO_3$; 4.8 g/l Hepes; pH 7.2), labelled and transported on ice to the laboratory within one hour and worked up immediately. The non-transformed human colon epithelium was separated from the tissue by perfusion-supported mechanical disaggregation as described previously [27] whereas the tumorous tissue was carefully prepared using a scalpel.

Isolation of single cells and determination of viability

Single cells were isolated from the normal and tumorous epithelial stripes by mincing and incubating in 3 ml HBSS (60 min, 37°C) which was supplemented with 6 mg proteinase K (Sigma; Steinheim, Germany) and 3 mg collagenase P (Boehringer; Mannheim, Germany). The suspensions of primary human colon cells were diluted with HBSS, centrifuged and resuspended in PBS (phosphate buffered saline; 8 g/l NaCl; 1.44 g/l Na_2HPO_4; 0.2 g/l KCl; 0.2 g/l KH_2PO_4; pH 7.3). Viabilities of the two different cell types were determined with the trypan blue exclusion test.

Incubation of tissue stripes

Small intact tissue stripes both normal and tumorous were incubated for 12 h for gene expression studies following a protocol as described previously [21]. Here, approximately 300 mg of tissue were placed into Petri dishes and after allowing attaching to the plastic surface for 15 min the tissues were treated with cell culture medium or with 10 mM butyrate diluted in cell culture medium. The medium consisted of minimal essential medium (MEM) with Earle's salts enriched with 20% FCS, 2 mM glutamine, 1% penicillin/streptomycin, 100 µg/ml gentamycin, 2.5 µg/ml fungizone, 10 ng/ml EGF, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite according to Rogler et al [28].

RNA Isolation and cDNA synthesis

For basal gene expression studies, small tissue pieces were immediately shock frozen with liquid nitrogen after preparation and were mechanically homogenized using a mortar and a pestle. After treatment with butyrate for 12 h the treated epithelial stripes were washed in PBS and were also shock frozen and grinded. The resulting tissue powder was resuspended in lysis buffer and total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA was eluted in 40 µl RNase free water and was stored at –20°C until use. The ratio $A^{260}/_{280}$ and the total concentration were determined spectrophotometrically (NanoDrop®, Peqlab Biotechnology, Erlangen, Germany). Additionally, formaldehyde denaturing RNA gel electrophoresis was carried out to check the integrity of the ribosomal RNA and exclude remaining DNA contamination.

For real-time PCR, up to one µg of total RNA was reverse transcribed using SuperScript II, First-Strand cDNA Synthesis System (Invitrogen) in 20 µl buffer with oligo-$(dT)_{15}$ primers according to manufacturers instructions.

Analysis of basal gene expression using cDNA macroarrays

Each of the two cDNA gene arrays contained 112 genes (3 blanks, 13 reference spots, and 96 pathway specific human genes; GEArray Q Series Human drug metabolism HS11 and Human Stress&Toxicity Gene Array HS12, SuperArray® Bioscience Corporation; Frederick, MD, USA). Genes were classified into functional categories, representing genes belonging to Phase I, Phase II and Phase III of biotransformation (HS11) and to Proliferation/Carcinogenesis, Growth Arrest/Senescence, Inflammation and Necrosis/Apoptosis (Oxidative & Metabolic Stress, Heating Stress, DNA Damage & Repair and Apoptosis Signaling), HS12. Detailed gene lists are available at the company's website (www.superarray.com). The array was performed according to the manufacturer's protocol and as has been previously described [21]. Briefly, single stranded cDNA was synthesized from total RNA (1 µg) *in vitro* which was labelled with dUTP-biotin. The cDNA macroarray membranes were hybridized overnight at 60°C with the biotin labelled cDNA probe. The hybridized membrane was subjected to chemiluminesence analysis for quantification of the conjugation-signals with streptavidin-linked alkaline phosphatase and CDPstar. The resulting signals were recorded with a CCD camera (Fujifilm LAS-1000, Diana, USA) and analyzed with AIDA array analysis (Raytest GmbH, Germany) software. Raw data were normalized between 0 and 100% expression by setting the signals of the means of the negative controls (areas without spotted gene sequences or with genes not expressed in human cells) to equal 0% and the means of the signals of the positive controls (household genes) to equal 100%. Thus, the data shown here represent mean expression levels relative to negative and positive reference genes.

Real-time PCR analysis for selected genes

Fifty nanograms cDNA calculated as RNA equivalents were used in a 25 µl PCR amplification reaction containing 2x iQ SYBR Green supermix® (100 mM KCl, 40 mM Tris-

HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, stabilizers) and 10 pmol gene specific primers for the target genes and reference (*hGAPDH*) gene. The following primer sequences were used for amplification:

GSTP1_F	5' ctg cgc atg ctg ctg gca gat c 3'
GSTP1_R	5' ttg gac tgg tac agg gtg agg tc 3'
GSTM2_F	5'agc cgt atg cag ctg gcc aaa c 3'
GSTM2_R	5'gga caa agg tga tct tgt ccc ca 3'
CAT_F	5' tgg aca agt aca atg ctg ag 3'
CAT_R	5' tta gga tga acg cta ag 3'
COX2_F	5' tcc tcc tgt gcc tga tga ttg c 3'
COX2_R	5' act gat gcg tga agt gct ggg 3'

PCR cycles included 1 cycle of 95°C for 2 min followed by 40 cycles each of 94°C for 30 s, a primer specific annealing temperature of 57-62°C for 30 s, and 72°C for 40 s, and a final extension step of 72°C for 10 min. Product-specific amplification was confirmed by melting curve analysis. All experiments were performed in duplicates. The fluorescence threshold value (C_T) was calculated using the iCycler iQ® optical v3.0a system software. The relative quantification of the target-*mRNA* expression was calculated with the comparative $\Delta\Delta C_T$ ($\Delta\Delta C_T = \Delta C_{T\ control} - \Delta C_{T\ reference}$) method. The fold change was calculated according to the efficiency method (E=2; fold change=$E^{difference}$)[29;30].

Datamining using the dbEST database

Expression data of single genes in the dbEST database provided on the NIH cGAP page ("Virtual Northern" function) can be obtained via http://cgap.nci.nih.gov/Genes/Gene-Finder. The search routine for checking all annotated human genes can be accessed via http://www.embl-heidelberg.de/~altenber/gemuend/Program [31;32]. This page allows searches in databases that contain cDNA and EST data and has access to approximately four

million ESTs and genes. The database collects gene expression data from other libraries and unifies them. In one of its subfunctions, “Virtual Northern”, gene expression data for 51 normal tissues and their corresponding cancerous counterparts are summarised. For specific genes, expression in these tissues is provided in a statistically pre-evaluated manner. Here, datamining was performed for colon cancer and corresponding normal tissue on the genes which were evaluated in vitro on the gene arrays.

Statistical analysis

Statistical evaluation was performed with the GraphPad Prism Version 4.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and Microsoft Office Excel 2003. Means were calculated from at least three independently reproduced experiments. Data were analysed for statistical significant differences using paired t-test which is indicated in the legends of the figures and tables.

Results

Viability of normal and tumor cells after isolation

Both cell types were successfully isolated from the two tissue specimens. Viability did not differ between normal (90.0±1.8) and tumor cells (91.7±2.2; mean±SD, paired t-test).

Comparison of basal gene expression between normal and tumor tissue from the same donor

For basal gene expression studies, RNA of normal and tumor cells isolated from tissue of four different donors was used for two pathway specific macroarrays. Table 1 shows that in the category of drug metabolism 19 genes of 96 were significantly differently expressed in tumor tissue compared to the normal corresponding tissue. Of these genes, 5 were up-regulated in tumor tissue whereas 14 were detected at a lower expression level in the tumor cells. Nine of these genes were also evaluated in data mining, five showed a very good correlation with the experimental data of the array analysis. Interestingly, GST alpha isoforms were found to be expressed more in tumor cells than in normal cells pointing to an enhanced metabolism in more progressed stages. In contrast, metallothioneins were lower expressed in tumor cells.

Using the array for oxidative and metabolic stress associated genes, only 10 of 96 genes were modulated. Of these, 5 were up-regulated in tumor cells, 5 were down-regulated. For seven genes, data mining was also performed again showing for five genes a quite good matching. Interestingly, in the four used donors genes belonging to proliferation and carcinogenesis were not found to be differentially expressed.

Effect of a butyrate treatment on normal and tumor cells using real-time PCR[1]

We have chosen GSTP1 and GSTM2 as members of important detoxifying enzyme systems and which have been described to be inducible in human colon cancer cell lines [15]. GSTP1 is 10fold more expressed in colon cells than GSTM2 (Figure 1). In normal cells, GSTP1 was induced by the butyrate treatment in two of the three donors whereas in tumor cells only one of 3 donors showed induction. The expression level of GSTP1 was slightly higher in the tumor cells compared to the corresponding normal cells. GSTM2 seemed to be more expressed in normal cells than in tumor cells, but was clearly induced in normal cells of only one donor. Tumor cells of all three donors were less responsive[2].

The expression levels of catalase were similar in normal and tumor cells (Figure 2). While the inducibility by butyrate in normal cells varied highly between different donors in the tumor cells catalase was slightly increased. As described in the literature, in the three donors COX-2 was at a higher expression level in the real-time analysis in tumor cells than in normal cells. In tumor cells of two donors, the butyrate treatment reduced COX-2 expression. In normal cells, cells from different donors showed different reactions to the butyrate treatment.

[1] Prior to submission: n size will be increased. COMT, MT1A and XRCC2 will be included in the PCR analysis, since they have given diverging results in experimental array analysis and in data mining. Statistical analysis based on higher "n" size will be performed.

[2] Prior to submission: The n size will be increased in the coming year, after which analysis of significance will be performed.

Discussion

The influence of dietary factors is studied extensively but a clear role of diet in colon cancer prevention still remains to be clarified. Interindividual heterogeneity affects gene expression level of detoxifying enzyme systems which determines the individual's sensitivity towards colon cancer risk factors. Differences in the genes themselves (e.g. polymorphisms in the gene or the promoter of a gene) can lead to enzymes with different metabolizing capacity [33;34]. However, the analysis of only the genetic background is insufficient or even misleading since the transcriptional activities of available genes can confound phenotypical properties. Thus, diet may influence expression. This influence moreover may occur at different transformation stages of the colonic epithelium since undetected polyps or adenomas may occur next to healthy tissue. Thus, the incidence, the recurrence or the progression of colon carcinogenesis may be influenced by dietary fibre and its fermentation products. By treating both cells types with butyrate we characterized the effects of the fermentation product in cells of different stages of carcinogenesis on four important genes of detoxification. Apart from the fermentation products, dietary fibre also contributes to chemoprevention by decreasing faeces transit time, lowering pH and binding of colon carcinogens.

Since normal epithelial cells have a finite lifespan and are not immortalized like tumor cell lines they can not be used as easily as tumor cell lines for chemoprevention research. Thus, many effects of butyrate (and other nutritional compounds in general) are described for tumor cells and mostly refer to prohibition of the development or the progression of carcinogenesis. It would be even more important to elucidate mechanisms by which the initiation of healthy normal cells can be prevented and thus the process of carcinogenesis can be blocked. To bridge the gap between the use of these different cell models we used both normal and tumor cells derived from the same donor. In particular, normal cells represent a more relevant model for cancer prevention.

The different effects by butyrate may reflect inherent differences in the cells, based on genetic differences which may or may not be connected to the transformation process but to the individual himself. The ability of cells to oxidize butyrate may influence their response to butyrate which can be seen in different effects of butyrate. The rate of removal of butyrate from the cytoplasm can therefore influence the availability of butyrate to exert its effects. Thus, cells that oxidize butyrate poorly may have higher intracellular concentrations of butyrate leading to direct butyrate-mediated effects, such as inhibition of histone deacetylase [35] with subsequent changes in gene expression. The responses of cells to butyrate may also depend on the cells' state of activation [36]. Cancer cell lines, however, are not an ideal model to be compared with physiological effects in isolated normal cells, since they represent a selected population of colon cancer cells that have adapted to conditions *in vitro*. Therefore, we isolated normal and tumor cells from the same patients to exclude differences in gene expression due to the isolation process.

Butyrate up-take studies over 12 h showed that comparable amounts of butyrate were metabolized by primary normal human colon cells, LT97 adenoma and HT29 adenocarcinoma cells (unpublished results). Thus, the state of activation of the different cells may be responsible for different effects of butyrate leading to apoptosis and inhibition of proliferation in cancer cells whereas non-transformed cells were not impaired.

It is described in the literature that GSTP1 is overexpressed in colon tumors and is regarded as a marker protein in human colonic carcinomas. This overexpression in cancer cells may lead to drug resistence. In contrast to GSTP1 expression, our array studies revealed a higher expression level of GSTA isoforms in tumor cells compared to the corresponding normal cells. We did not detect an overexpression of GSTP1. The reason is maybe that this gene is normally expressed at a high level in colon tissue and it can not be excluded that due to the diagnosis colon cancer that the normal colon tissue may also undergo changes in gene expression [37]. The expression level of GSTP1 also varied between the four donors.

Moreover, we did not detect significant differences in genes belonging to signalling pathways of proliferation or carcinogenesis which we could not explain so far. Maybe the genes are in general only at a low expression level which is sufficient for signal transduction but thus, they may fall below detection limit of the array method. We also could not prove the described overexpression of COX-2 in colon cancer probably for the same reason. Contrarily, the gene expression data sets show a down-regulation of metallothioneins which was congruent to the data mining results. Of course, it will be necessary to compare expression levels in more donors.

In conclusion, the presented study describes differences in gene expression on an individual basis between normal and tumor cells. In general, the utilization of cell models representing early, middle and late stages of colon carcinogenesis will add more knowledge on mechanisms of dietary prevention of the colorectal transformation stage process. Even it necessitates complicated preparation and cell culture techniques using both normal and tumor cells from one donor the significance of dietary chemoprevention can be enhanced. Using this approach, genes can be identified which can be considered as targets in human colon carcinogenesis as well as genes which may represent targets in nutritional chemoprevention.

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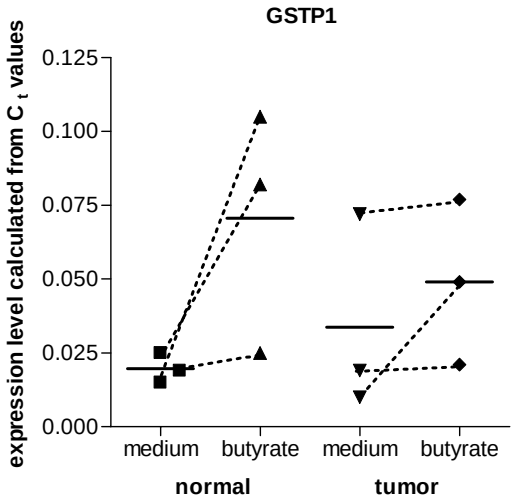
Figure 1

Effect of a butyrate treatment in normal and corresponding tumor cells derived from the same donor on expression of GSTP1 and GSTM2. Modulation of gene expression was analysed with real-time PCR and the basal gene expression levels were calculated from the C_t values.

Donor	fold change			
	normal		tumor	
	GSTP1	GSTM2	GSTP1	GSTM2
1	3,4	1,0	1,1	1,7
2	7,0	2,6	1,1	1,3
3	1,4	1,0	5,1	6,7



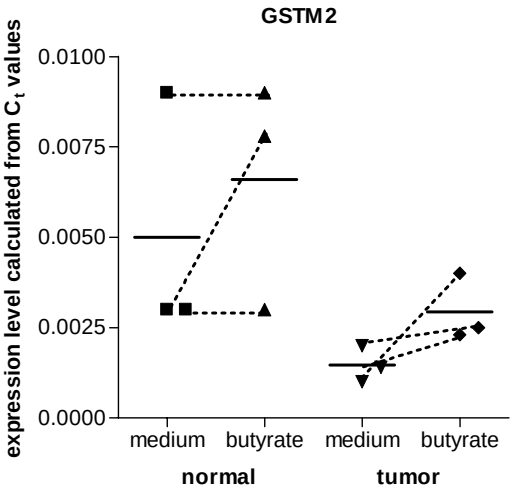
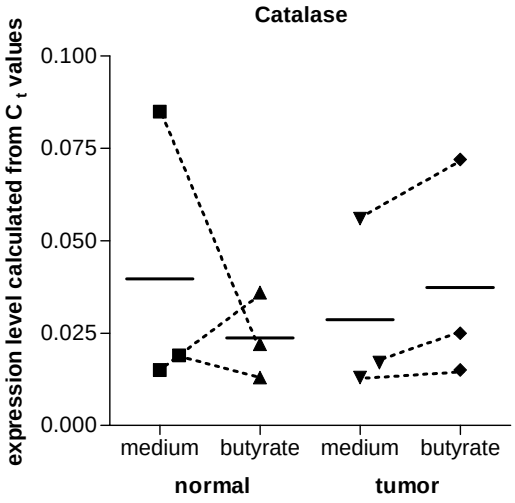


Figure 2

Effect of a butyrate treatment in normal and corresponding tumor cells derived from the same donor on expression of catalase and COX-2. Modulation of gene expression was analysed with real-time PCR and the basal gene expression levels were calculated from the C_t values.

Donor	fold change			
	normal		tumor	
	COX	CAT	COX	CAT
1	2,3	0,3	0,6	1,5
2	1,3	2,4	0,8	1,3
3	0,5	0,7	4,0	1,1



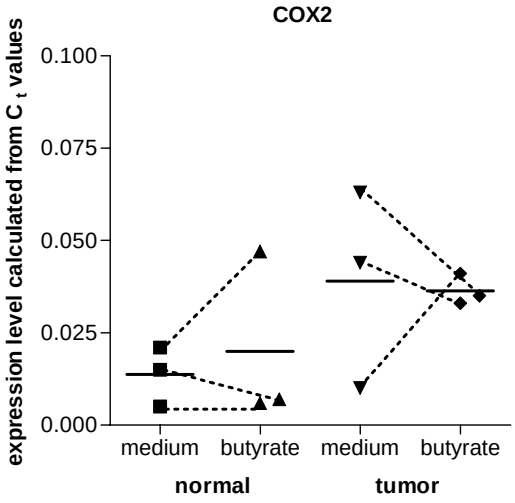


Table 1

Summary of genes which were differently expressed in tumor cells compared to normal cells. The table combines the results for drug metabolism and stress and toxicity array (n=4, means±standard deviation) as well as the results from data mining.

Array	Functional gene family	Number of expressed genes	Differentially expressed genes	Normal vs tumor tissue				paired t-test	fold change	fold change data mining	p-value data mining
				Normal tissue		Tumor tissue					
				means	SD	means	SD				
HS 11 Human Drug Metabolism	**Phase I, p450 Family**	7/25	CYP2F1	8,3	15,4	19,5	8,7		2.3	n.d.	
			CYP3A4	10,1	5,6	4,8	1,3		0.5	n.d.	
			CYP3A7	11,2	7,4	4,9	5,8		0.4	n.d.	
	Phase II, Acetyltransferases	8/10	CHAT	163,3	52,4	140,8	54,3	*		n.d.	
			CRAT	93,1	31,3	34,0	22,2		0.4	1.0	
			HAT1	2,1	5,7	14,1	7,8	*	6.7	up in cancer	
	Phase II, Glutathione S-transferases	8/12	GSTA2	4,4	3,8	9,3	2,8	**	2.1	n.d.	
			GSTA3	3,6	3,2	8,0	2,1		2.2	n.d.	
			GSTA4	-2,3	14,4	12,0	6,5		5.1	up in cancer	
			MGST3	63,7	11,4	23,9	11,5	*	0.4	0.35	
	Phase II, Sulfotransferases	7/21	SULT1B1	31,6	12,2	12,8	8,4	*	0.4	n.d.	
	Phase II, Miscellaneous	8/13	COMT	92,9	23,9	32,0	19,1	*	0.3	4.3	p=0.08
			MORF	14,3	7,5	5,9	2,3		0.4	n.d.	
			UGT2A1	10,7	6,1	3,0	3,6		0.3	n.d.	
	Phase III, Metallothioneins	8/8	MT1A	68,5	41,5	31,3	19,0		0.5	7.1	p=0.01
			MT1G	249,6	168,8	132,9	62,0		0.5	down	
			MT1L	322,5	177,1	185,3	110,2			n.d.	
			MTIX	601,2	357,7	268,5	175,3		0.4	down	
	Phase III, p-Glycoproteins	2/7	ABCG2	154,9	63,0	75,3	55,2		0.5	0.1	
HS 12 Human Stress & Toxicity	**Proliferation/ Carcinogenesis**	4/6									
	Growth Arrest / Senescence	1/8									
	Inflammation	5/14	IL18	127,6	47,1	80,2	59,7	*		n.d.	
	Oxidative & Metabolic Stress	14/22	EPHX2	393,4	262,6	257,8	139,6		0.7	0.7	
			GPX	6,2	14,5	18,0	13,2	*	2.9	1.8	
			MT1H	73,3	32,6	34,3	11,5		0.5	n.d.	
	Heating Stress	15/18	HSP105B	12,6	9,5	30,9	23,8		2.5	3.6	
			HSPCA	109,4	25,9	150,1	26,5	*	1.4	1.3	
			HSPCB	521,2	224,6	451,5	138,8			n.d.	
			HSPD1	12,2	10,0	37,8	29,4		3.1	3.4	
	DNA Damage & Repair	7/15	UGT1A9	20,6	7,7	6,8	2,4	*	0.3	0.93	p=0.47
			XRCC2	4,2	1,4	10,4	6,1		2.5	0.7	p=0.01
	Apoptosis Signaling	6/13									

3 Weitere Ergebnisse

3.1 Epithelzellnachweis

Dickdarmepithelzellen sind kontinuierlich einer komplexen Mischung an Substanzen ausgesetzt, die über die Ernährung und die Verdauung in die Fäzes gelangt und so in Kontakt mit der Dickdarmschleimhaut tritt (DeKok & van Maanen, 2000). Diese Nahrungsinhaltsstoffe können sowohl protektiv als auch potentiell karzinogen wirken. Insbesondere die epithelialen Kryptzellen stellen die Zielzellen der Kolontumorentstehung dar (Lipkin, 1975; Chang, 1984; Potten & Loeffler, 1990).
Um zu ermitteln, welchen Anteil die relevanten Epithelzellen in den präparierten Kolongeweben und Primärzellsuspensionen ausmachen, wurde ein Epithelzellnachweis durchgeführt. Dazu wurde der epitheliale Antikörper Ber-EP4 (Latza *et al.*, 1990) an magnetische Bead-Partikel gekoppelt. Die aus primärem Kolongewebe hergestellte Zellsuspension wurde mit den Antikörper-Bead-Komplexen inkubiert, wodurch es zu einer Antigen-Antikörper-Bindung zwischen dem epithelialen Antikörper und Epithelzellen kam. Anschließend wurde die Zellsuspension einem magnetischen Feld ausgesetzt, wodurch die Zell-Bead-Komplexe von anderen Zelltypen abgetrennt werden konnten. Mittels Neubauer-Zählkammer wurde die Zellzahl der Zell-Bead-Komplexe bestimmt sowie die Zellzahl der im Überstand verbliebenen Zellen.

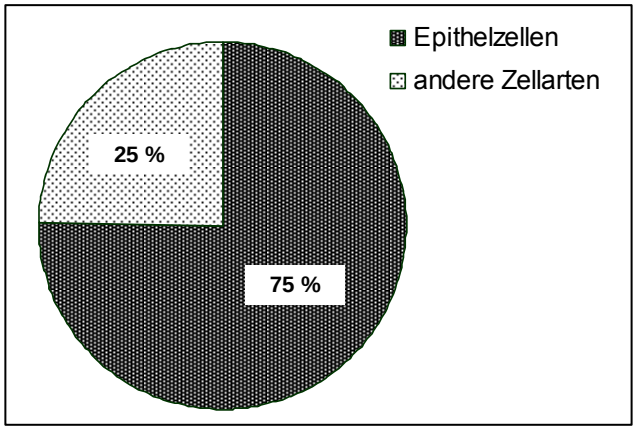


Abb. 4: Prozentualer Anteil an Epithelzellen in den Zellsuspensionen aus primären Kolongeweben (n=7).

Im Mittel von sieben unabhängig voneinander durchgeführten Kolongewebepräparationen der Epithelschicht (Schäferhenrich *et al.*, 2003), bestanden die Zellsuspensionen zu 75 % aus Epithelzellen (Abb. 4). Die übrigen

25 % setzten sich aus anderen Zelltypen, wie z.B. Fibroblasten oder Leukozyten, zusammen, die in der Darmwand enthalten sind.

Aus dieser Verteilung lässt sich ableiten, dass die für die hier dargestellten Experimente verwendeten Zellsuspensionen Aussagen über die Wirkung von Fermentationsprodukten auf die relevanten Zielzellen der Kolonkarzinogenese (Lipkin, 1975; Chang, 1984; Winawer, 1999) zu lassen und die methodisch bedingte Verunreinigung mit anderen Zellarten vernachlässigbar sein sollte.

3.2 Vorversuche zur Vitalitätsbestimmung nach Inkubation der primären Kolonzellen mit Fermentationsprodukten

Im Rahmen dieser Arbeit sollte eine Inkubationsdauer definiert werden, nach der primäre Kolonzellen eine ausreichende Vitalität aufweisen und intakte RNA für Genexpressionsuntersuchungen isolierbar ist. Diese Inkubationsdauer sollte lang genug sein, um Modulationen der Genexpression erfassen zu können.

In Voruntersuchungen wurden primäre Kolonzellen als Einzelzellen in Kollagen A-beschichtete Zellkulturgefäße ausgesät (Rogler *et al.*, 1998). Nach 4 und 12 Stunden wurde die Vitalität der Zellen mittels Trypanblau-Ausschlusstest bestimmt. Abb. 5 zeigt, dass im Vergleich zur Ausgangsvitalität die Vitalität zeitabhängig nach 4 und 12 Stunden abnahm.

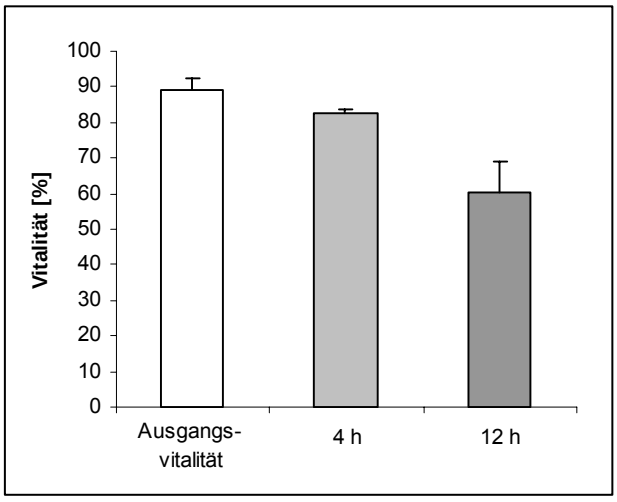


Abb. 5: Vitalität primärer Kolonzellen nach 4 und 12 Stunden in Zellkultur (Mittelwert ± Standardabweichung; n=4).

Wurden dagegen primäre Epithelstreifen für 12 Stunden inkubiert, ließen sich höhere Vitalitäten erzielen. Auch die Behandlung mit Fermentationsprodukten hatte keinen signifikanten Einfluss auf die Zellvitalfunktion (Abb. 6).

Für weitere Experimente wurden intakte Epithelstreifen eingesetzt, aus denen nach einer Inkubationszeit von 12 Stunden RNA oder Cytosol isoliert werden konnte. Diese Voruntersuchungen bestätigen, dass für das Überleben primärer Kolonzellen der Zell-Zell-Kontakt eine große Bedeutung besitzt (Strater *et al.*, 1996; Grossmann *et al.*, 2003).

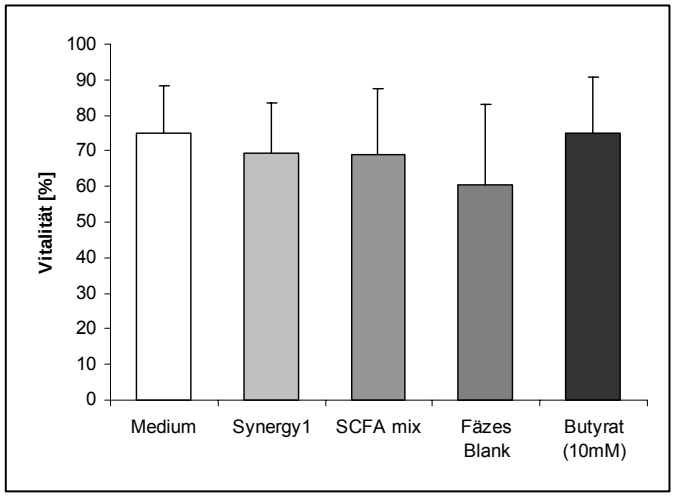


Abb. 6: Vitalität primärer Kolonzellen nach Inkubation als Epithelstreifen mit Fermentationsprodukten (Mittelwert ± Standardabweichung; n=6). Die Mischung kurzkettiger Fettsäuren (SCFA mix) spiegelt die Konzentration kurzkettiger Fettsäuren wider, welche nach Fermentation des Fruktans Synergy1® (ORAFTI) im Überstand messbar waren. Der Fäzes Blank stellt einen Fermentationsüberstand ohne Substratzugabe dar.

3.3 Ermittlung genetischer Polymorphismen

Polymorphismen in Genen, die für Entgiftungsenzyme kodieren, können, wenn sie katalytisch relevante Regionen betreffen, die Enzymaktivität und damit die zelluläre Entgiftungskapazität beeinträchtigen. Solche Veränderungen könnten individuelle Unterschiede in der Kapazität des Fremdstoffmetabolismus erklären und wurden hier für vier GST-Isoformen und Katalase untersucht.

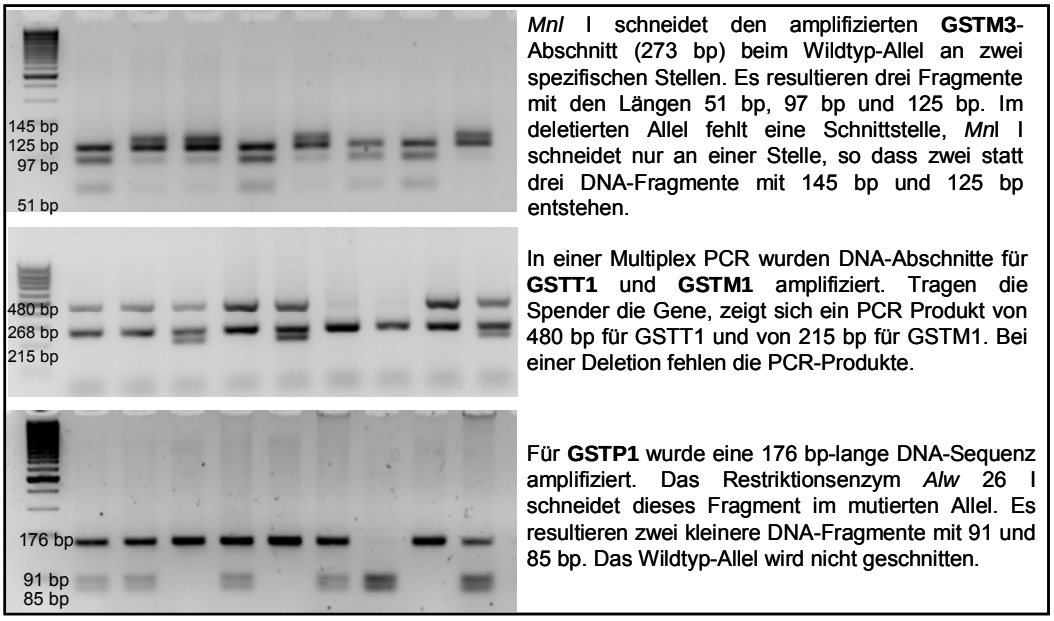
Ein Deletionspolymorphismus führt zum vollständigen Verlust der jeweiligen Enzymexpression. Die Nullpolymorphismen der GSTM1 und GSTT1 können mittels Multiplex PCR detektiert werden (Hayes & Strange, 2000). Hierbei werden in einer Reaktion mehrere Fragmente durch Verwendung spezifischer Primerpaare amplifiziert. Für diese beiden Gene wurden die Fragmente für GSTT1 mit 480 Basenpaaren (bp) und für GSTM1 mit 215 bp gebildet. Als interne Kontrolle wurde ein Fragment (268 bp) des β-Globin-Gens co-amplifiziert, um den Erfolg der PCR zu verfolgen, falls beide GST-Gene deletiert sind.

Im GSTP1 Gen ist ein Polymorphismus bekannt, der durch Punktmutation im Nukleotid 313 (A→G Transition) entsteht. Eine Punktmutation kann z.B. mit dem Austausch einer Aminosäure einhergehen, wodurch es zu einer Veränderung der

Enzymaktivität kommen kann. Diese kann gesteigert oder vermindert werden. Die Substratspezifität kann sich ändern, wobei die Aktivität gleich bleiben kann.

Für GSTM3 wurde eine 3-Basenpaar-Deletion beschrieben. Diese Polymorphismen können nach Amplifikation des Genabschnittes in einer PCR mit Hilfe von Restriktionsenzymen detektiert werden (Eaton & Bammler, 1999). Ein Restriktionsenzym ist eine Endonuklease, die spezifische Sequenzen in der DNA erkennt und sie schneidet. Durch eine Auftrennung der entstandenen Fragmente in einem Agarosegel kann die Punktmutation nachgewiesen werden.

Von 42 in dieser Arbeit untersuchten Individuen wurden 30 auf diese vier GST-Genotypen hin untersucht. Dies sollte Aufschluss darüber geben, ob unterschiedliche Genotypen die aufgetretenen interindividuellen Variationen auf Genexpression und Enzymaktivität klären können. Abb. 7 zeigt beispielhaft Gelbilder der detektierten Polymorphismen.



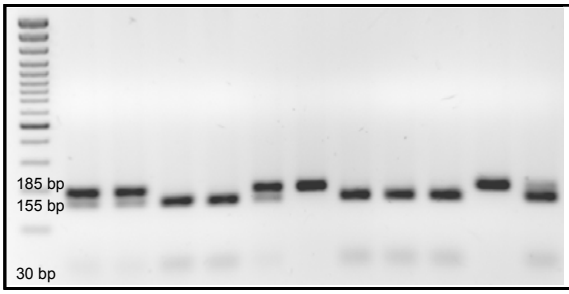
Mnl I schneidet den amplifizierten **GSTM3**-Abschnitt (273 bp) beim Wildtyp-Allel an zwei spezifischen Stellen. Es resultieren drei Fragmente mit den Längen 51 bp, 97 bp und 125 bp. Im deletierten Allel fehlt eine Schnittstelle, *Mn*l I schneidet nur an einer Stelle, so dass zwei statt drei DNA-Fragmente mit 145 bp und 125 bp entstehen.

In einer Multiplex PCR wurden DNA-Abschnitte für **GSTT1** und **GSTM1** amplifiziert. Tragen die Spender die Gene, zeigt sich ein PCR Produkt von 480 bp für GSTT1 und von 215 bp für GSTM1. Bei einer Deletion fehlen die PCR-Produkte.

Für **GSTP1** wurde eine 176 bp-lange DNA-Sequenz amplifiziert. Das Restriktionsenzym *Alw* 26 I schneidet dieses Fragment im mutierten Allel. Es resultieren zwei kleinere DNA-Fragmente mit 91 und 85 bp. Das Wildtyp-Allel wird nicht geschnitten.

Abb. 7: Beispielbilder der Genotypisierung verschiedener Spender von Dickdarmgewebe. Die erste Spur von links zeigt den DNA-Größenstandard, wobei eine Bande einer Länge von 100 bp entspricht. Jede Spur zeigt das PCR-Ergebnis eines anderen Spenders.

Für das Katalase-Gen ist ein Promotor-Polymorphismus beschrieben, der die Enzymaktivität beeinflusst. Träger des Wildtyp-Promotors besitzen eine höhere Aktivität als Individuen mit einem mutierten Promotor (Ahn *et al.*, 2006). Insgesamt 13 der bearbeiteten Gewebeproben wurden auf diesen Polymorphismus hin untersucht (Abb. 8).



Die spezifischen Primer amplifizieren ein 185 bp großes Fragment, welches im Wildtyp Allel in 155 und 30 bp Fragmente geschnitten wird. Durch die Mutation [-262 C→T] fällt diese Schnittstelle für das Restriktionsenzym *Sma* I weg (Forsberg *et al*., 2001).

Abb. 8: Beispielgel der Genotypisierung hinsichtlich des Katalase-Promotor-Polymorphismus. Die erste Spur von links zeigt den DNA-Größenstandard, wobei eine Bande einer Länge von 100 bp entspricht. Jede Spur zeigt das PCR-Ergebnis eines anderen Spenders.

Durch Bestimmung dieser vier GST-Polymorphismen bzw. des Katalase-Promotor-Polymorphismus konnten jedoch interindividuelle Variationen hinsichtlich der Expressionshöhe und der Modulierbarkeit dieser Gene nicht geklärt werden, da einerseits unterschiedliche Genexpressionshöhen und -änderungen bei gleichen Genotypen auftraten, andererseits vergleichbare Modulationen bei unterschiedlichen Genotypen erfasst wurden. Um diesbezüglich eine Aussage zu treffen, ist die Untersuchung eines größeren Patientenkollektivs notwendig.

4 Diskussion

4.1 Das Primärzellmodell

Normale Kolonepithelzellen haben eine begrenzte Lebensdauer. Eine zelluläre Immortalisation benötigt Mutationen in wichtigen Kontrollproteinen des Zellzyklus und entwickelt sich über mehrere Jahrzehnte hinweg (Fenton & Hord, 2006). Im Kolonepithel sind nur die Stammzellen teilungsfähig, die sich im unteren Drittel der Krypten befinden. Es gestaltet sich daher besonders schwierig, diese Zellen in Kultur zu bringen, da der größte Anteil der Epithelzellen bereits ausdifferenziert ist und *in vivo* nur eine Lebensdauer von etwa 5 Tagen hat. Der Isolationsprozess primärer Zellen aus dem Gewebeverband heraus kann zudem dazu führen, dass die Zellen spontan in Apoptose gehen (Pedersen *et al.*, 2000; Grossmann *et al.*, 2003). Die in der Literatur beschriebenen Primärzellkulturansätze versuchen, dieser Problematik Rechnung zu tragen. Um den Zellen Kontakt zur extrazellulären Matrix zu gewährleisten, werden häufig Kollagen-beschichtete Zellkulturgefäße benutzt (Rogler *et al.*, 1998; Pedersen *et al.*, 2000). Außerdem wurden verschiedene Zellkulturmedien zum Einsatz gebracht. Auch wenn die publizierten Methoden eine Kultivierbarkeit der primären Kolonepithelzellen von einigen Tagen versprechen, finden diese Systeme bislang keine breite Anwendung (Rogler *et al.*, 1998; Pedersen *et al.*, 2000; Grossmann *et al.*, 2003).

Für die hier dargestellten Experimente wurden primäre Kolonzellen aus normalem Dickdarmgewebe isoliert. Für einige Experimente wurde aus dem Epithel eine Einzelzellsuspension hergestellt (***Publikation I***). Diese Suspension, die neben vereinzelten Zellen auch Krypten oder Kryptbruchstücke enthielt, konnte jedoch nur kurzzeitig in Kultur gehalten werden. So zeigten die Untersuchungen, dass die primären Kolonzellen nur bis zu zwölf Stunden *in vitro* behandelt werden konnten, ohne zu stark in ihren Vitalfunktionen beeinträchtigt zu werden (***Publikation III, IV***). Wurden intakte Epithelstreifen, also Zellen im Verband, für die Versuche eingesetzt, konnte eine im Mittel 10 % höhere Vitalität nach einer zwölfstündigen Inkubation erzielt werden (***Publikation II, III, IV***). Für die durchgeführten Genexpressionsuntersuchungen wurden zwölf Stunden als Inkubationsdauer ausgewählt, da nach dieser Zeit noch ausreichend vitale Zellen mit intakter RNA isoliert werden konnten. Zudem sollte diese Zeitspanne ausreichen, einen Einfluss der Inkubationen auf die Genexpression zu ermitteln (Knoll *et al.*, 2005).

Da sich Adenokarzinomzelllinien weitaus besser kultivieren lassen, wurden Untersuchungen zu Mechanismen der Chemoprävention bislang zumeist an derartigen Zellen durchgeführt (Richter *et al.*, 2002), was wichtige Einblicke in molekulare Mechanismen von Präventionsmöglichkeiten in veränderten Zellen ermöglicht. Wenn jedoch der Einfluss von Nahrungsinhaltsstoffen auf die Fähigkeit hin untersucht werden soll, die Entstehung von Krebs zu verhindern oder eine Progression präneoplastischer Zellen zu beeinflussen, ist die Relevanz von Tumorzelllinien fraglich. Die Effekte von Butyrat liefern nur ein Beispiel dafür, dass die Wirkung von chemopräventiven Agenzien vom Grad der zellulären Transformation abhängt (Gibson *et al.*, 1999).

Da Schätzungen zu Folge 30-40 % der Krebserkrankungen einen direkten Ernährungsbezug haben (World Cancer Research Fund, 1997), besitzt die Ernährung daher womöglich den größten Einfluss, bevor eine Entartung stattgefunden hat. Es ist daher von besonderer Bedeutung, Mechanismen in normalen Zellen zu finden, durch welche diese besser vor einer Entartung geschützt werden. Der Nachweis des epithelialen Ursprungs unter Verwendung des Antikörpers Ber-EP4 (Latza *et al.*, 1990) zeigte, dass die isolierten primären Kolonzellen zu 75 % epithelialen Ursprungs waren. Bei den restlichen Zellen handelt es sich vermutlich hauptsächlich um Fibroblasten und Blutzellen.

Primäre Kolonepithelzellen stellen somit ein wichtiges Zellmodell dar, um modulierende Effekte von Nahrungsinhaltsstoffen in den Zielzellen der Kolonkarzinogenese zu untersuchen (Lipkin, 1975; Winawer, 1999) und Aussagen über Möglichkeiten einer Primärprävention machen zu können.

4.1.1 Interindividuelle Variation

Im Gegensatz zu Experimenten mit Zelllinien hingen die Untersuchungen im Rahmen dieser Arbeit von der Bereitstellung des humanen Kolongewebes ab, das aus medizinisch notwendigen Kolonresektionen stammte. Aufgrund der begrenzten Lebenszeit der Zellen musste für jeden Versuch das Gewebe eines neuen Spenders verwendet werden. Nach der Operation wurden spenderspezifische Daten zur Verfügung gestellt und es wurden einige GST-Polymorphismen (Bell *et al.*, 1993; Pemble *et al.*, 1994; Harries *et al.*, 1997; Jourenkova-Mironova *et al.*, 1998) bzw. ein Polymorphismus im Katalase-Promotor (Forsberg *et al.*, 2001) bestimmt (***Kapitel 3.3***). Trotz Abgleich mit diesen Parametern trat eine starke interindividuelle Variation hinsichtlich der Genexpression auf. Mögliche Ursachen dafür könnten kurzfristig die

Einnahme von Medikamenten oder der Verlauf der Operation sein (Geokas *et al.*, 1985). Längerfristig oder sogar lebenslang beeinflussen Ernährung und Lebenswandel („Life-Style Faktoren“) des Einzelnen sowie die genetisch definierte Ausstattung der Zellen die Genexpression (Augenlicht & Heerdt, 1992; Yang *et al.*, 2005).

4.1.2 Erfassung der Sensitivität von primären Kolonzellen gegenüber Risikofaktoren der Ernährung

In den hier dargestellten Untersuchungen zur Genotoxizität vom Hämin und Hämoglobin konnte gezeigt werden, dass diese aus der Ernährung stammenden eisenhaltigen Komponenten genotoxisches Potential in Kolonzellen besitzen (***Publikation I***). Nicht resorbiertes Eisen aus rotem Fleisch könnte im Dickdarm über die Haber-Weiss- und Fenton-Reaktion zur Bildung von freien Radikalen aus Peroxiden führen, welche einen Risikofaktor für die Kolonkarzinogenese darstellen. Ein mögliches Ziel einer Ernährungsintervention könnte daher beinhalten, die Abwehrkapazität in gesunden Zellen gegenüber toxischen Stoffen zu steigern. Eine Ernährung reich an Obst und Gemüse, die zahlreiche antioxidative Inhaltsstoffe enthält, könnte dazu beitragen, die durch Eisen gebildeten freien Radikale abzufangen, bevor diese z.B. die Basen der DNA, Proteine oder Lipide verändern können (Pool-Zobel *et al.*, 1998). Die Bildung freier Radikale steht in engem Zusammenhang zur Ernährung. Dies zeigten Messungen von freien Radikalen in Fäzeswässern von Probanden, die eine hohe Menge an rotem Fleisch und Fett verzehrten. Die Konzentration von ROS nahm dagegen deutlich ab, wenn die Ernährung durch pflanzliche Lebensmittel geprägt war (Erhardt *et al.*, 1998).

Für Untersuchungen zur Genotoxizität eignen sich primäre Kolonzellen, da durch Messung induzierter DNA-Strangbrüche einzelne Risikofaktoren auf ihr Potential hin getestet werden können, einen möglichen Einfluss auf die Initiation nehmen zu können. So wurden auch andere genotoxische Substanzen (H_2O_2, HNE, 2-Dodecylcyclobutanon), die in der Ernährung vorkommen, an diesem Zellmodell getestet (Pool-Zobel & Leucht, 1997; Pool-Zobel *et al.*, 1999; Schäferhenrich *et al.*, 2003; Oberreuther-Moschner *et al.*, 2005; Knoll *et al.*, 2006).

4.2 Basale Expression von Entgiftungsenzymen

Ernährungsgewohnheiten und „Life-Style“ haben einen großen Einfluss auf das Risiko, an Dickdarmkrebs zu erkranken (Cassidy *et al.*, 1994). Die Induktion von

Entgiftungsenzymen wie GST könnte eine Möglichkeit darstellen, das Risiko für Kolonkrebs zu reduzieren, da die Exposition gegenüber Risikofaktoren durch diese Enzymsysteme herabgesetzt werden kann (Ebert *et al.*, 2001). In früheren Studien wurde bereits die basale Proteinexpression von GST untersucht (Ebert *et al.*, 2003). Hierbei zeigte sich, dass die Expression zwischen einzelnen Spendern stark variierte. Aufgrund dieser unterschiedlichen Expressionsmuster könnten einzelne Individuen mit deutlich verringerter GST-Expression einem höheren Risiko ausgesetzt sein.

Die Expression und Aktivität von Entgiftungsenzymen ist insbesondere in primären Kolonzellen von Bedeutung, um die noch teilungsfähigen Stammzellen vor genetischen Schädigungen zu schützen (Potten & Loeffler, 1990) bzw. ausdifferenzierte Tochterzellen vor Mutationen zu bewahren, welche ihnen eine erneute Teilungsfähigkeit ermöglichen könnten (Kinzler & Vogelstein, 1996).

Durch die Weiterentwicklung der Zellkulturtechniken konnte auch in primären Zellen die GST-Expression auf transkriptioneller Ebene, sowie eine mögliche Modulation durch Fermentationsprodukte untersucht werden. Die Ergebnisse der basalen GST-Expressionsanalyse spiegelten das gewebespezifische Expressionsmuster für GST wider (***Publikation II***), indem GSTP1 und GSTT2 besonders stark exprimiert wurden. Für die meisten GST (GSTA, GSTP1, GSTT2) war die basale Expression der einzelnen Spender vergleichbar, während für einige eine deutliche Streuung auftrat (GSTM2, GSTM5). So variierte die GST-Expression zwischen dem Spender mit der höchsten GST-Expression und der Expression des Spenders mit der geringsten Expression um 100 %. Daraus lässt sich schlussfolgern, dass die Expression von Entgiftungsenzymen im normalen Gewebe einer Modulation durch zahlreiche endogene und exogene Faktoren unterliegt.

Ferner zeigten diese Untersuchungen, dass die Kultivierung der primären Kolonzellen für zwölf Stunden bereits zu einer veränderten Genexpression im Vergleich zu der des basalen Gewebes zum Zeitpunkt null führte. Außerdem unterschied sich die GST-Expression in primären Zellen deutlich von der in Tumorzelllinien, wodurch unterschiedliche Sensitivitäten und Reaktionen bedingt sein können. So lag die Expression der GSTM3, GSTM5, GSTT2 und der mikrosomalen GST in den Zelllinien auf einem niedrigeren Niveau (***Publikation II***).

Durch die Verbesserung der Zellkulturmethodik wurde es möglich, primäres Kolongewebe bis zu zwölf Stunden mit Butyrat und anderen komplexen

Fermentationsüberständen zu behandeln und so die Modulation der Genexpression zu untersuchen.

4.3 Modulation von Entgiftungsenzymen durch Darmfermentationsprodukte

Buttersäure als viel untersuchtes Darmfermentationsprodukt aus Ballaststoffen im Kolon besitzt die Fähigkeit, GST zu aktivieren (Ebert *et al.*, 2001). Dem könnten eine Modifizierung der Histonacetylierung zugrunde liegen (Kiefer *et al.*, 2006) oder Änderungen der MAP-Kinase-Kaskade (Ebert *et al.*, 2001). In vergleichenden Experimenten zum Einfluss von Buttersäure wurde bestätigt, dass dieses Fermentationsprodukt in allen drei verwendeten Zelltypen unterschiedlichen Transformationsgrades (HT29 Adenokarzinom-, LT97 Adenom- und Primärzellen) als Induktor für GST wirkte (***Publikation II***). Die Induktion von GSTT2 und GSTA Isoformen durch Butyrat in primären Kolonzellen könnte für die Entgiftung von HNE, Dibenzopyren-diol-epoxid und Hydroperoxiden von Bedeutung sein (Eaton & Bammler, 1999; Hayes *et al.*, 2005). Butyrat stellt somit zum einen eine Energiequelle für primäre Kolonzellen dar (Roediger, 1989; Scheppach *et al.*, 1992), zum anderen übt es auch in nicht-transformierten Zellen einen modulierenden Einfluss auf die Genexpression aus.

Auch wenn zahlreiche Untersuchungen insbesondere der Buttersäure als Darmfermentationsprodukt eine zentrale Rolle als protektiven Faktor zusprechen (Wachtershauser & Stein, 2000; Sengupta *et al.*, 2006), spiegeln komplexe Fermentationsüberstände die Bedingungen *in vivo* besser wider. Diese Fermentationsüberstände enthalten zum einen ein Gemisch aus verschiedenen kurzkettigen Fettsäuren, zum anderen aber auch Amine, Sulfate, weitere bakterielle Produkte und Gallensäuren (Cummings & Englyst, 1987; McGarr *et al.*, 2005). Die Beschaffenheit der Fermentationsprodukte wird maßgeblich durch die Zusammensetzung der Mikroflora beeinflusst (Wang & Gibson, 1993; Klinder *et al.*, 2004b). Daher kann der Einsatz unterschiedlicher Fäzesproben für eine *in vitro* Fermentation die biologischen Wirkungen des generierten Überstandes beeinflussen. Auch die Beeinflussung der Mikroflora des Dickdarms, beispielsweise durch Präbiotika, verändert die Zusammensetzung und damit die Wirkungen der Fermentationsüberstände (Klinder *et al.*, 2004b).

Die *in vitro* Fermentation von Inulin (Synergy1®) steigerte die Konzentration an kurzkettigen Fettsäuren im Überstand auf das nahezu Dreifache (94,4 mmol/L; ***Publikation III***) im Vergleich zur Fäzeskontrolle. Dieses Ergebnis reflektiert mögliche

Konzentrationen und molare Verhältnisse, wie sie nach Verzehr hoher Mengen an Ballaststoffen messbar sind (Cummings, 1981).

Mehrere *in vitro* und *in vivo* Tierstudien, wie ausführlich in ***Publikation V*** dargestellt, konnten protektive Effekte auf die Kolonkrebsentstehung nach Inulingabe zeigen. So wurde die Entstehung von ACF gehemmt und die Entstehung von Tumoren reduziert (Femia *et al.*, 2002; Klinder *et al.*, 2004a; Klinder *et al.*, 2004b). Bislang existierten jedoch keine Daten für den Einfluss von Inulin (oder des daraus resultierenden Fermentationsprofils) auf humane, nicht-transformierte Kolonzellen. Im Gegensatz zu einer wachstums-inhibierenden Wirkung auf Tumorzellen tolerierten primäre Kolonzellen auch hohe Konzentrationen des Fermentationsüberstandes (***Publikation III***). Die Abnahme der metabolischen Aktivität der Zellen stellte einen zeitabhängigen Vorgang dar, der nicht durch höhere Konzentrationen des Fermentationsüberstandes verstärkt wurde. Im Gegenteil förderte der Fermentationsüberstand aus Inulin die metabolische Aktivität der Zellen, was auf trophische Wirkungen hindeutet.

Im Vergleich zur Mediumbehandlung veränderte bereits der Fäzes-Kontrollüberstand (Fermentation ohne Substrat) die Expression von Genen, die für Biotransformationsenzyme kodieren, was auf den Einfluss anderer Substanzen als kurzkettige Fettsäuren in den Fäzes schließen lässt. Bei diesen Substanzen handelt es sich womöglich neben bestimmbaren Gallensäuren um bislang nicht aufgeklärte bakterielle Abbauprodukte (Roberfroid, 2005). Die Butyratkonzentration des für die Experimente verdünnten Fermentationsüberstandes betrug nur etwa 1 mM, daher könnten weitere kurzkettige Fettsäuren und andere Produkte im komplexen Fermentationsüberstand zur Modulation der Genexpression beitragen (Beyer-Sehlmeyer *et al.*, 2003). GSTM2 und GSTM5 wurden induziert, was zu einer verbesserten Entgiftung von elektrophilen Zwischenprodukten aus Karzinogenen bzw. Umweltgiften führen kann (Hayes *et al.*, 2005). Zusätzlich besitzen die GSTM-Isoformen eine Peroxidaseaktivität, wodurch Produkte des oxidativen Stresses abgebaut werden können (Hayes & McLellan, 1999). Im Gegensatz dazu wurde die Expression von CYP7A1 gesenkt. Dies könnte eine reduzierte metabolische Aktivierung von Xenobiotika durch Monooxygenasen nach sich ziehen. Die Induktion von zwei Sulfotransferasen könnte auch die zelluläre Entgiftung von phenolischen Verbindungen und Katecholen fördern (Carlini *et al.*, 2001). Insgesamt können diese Veränderungen als chemoprotektiv eingestuft werden, da sie zur Reduktion der Exposition von Karzinogenen führen könnten. Die Veränderungen auf

transkriptioneller Ebene konnten allerdings bisher noch nicht auf Enzymaktivitätsebene bestätigt werden. Gründe dafür könnten sein, dass die am stärksten modulierte GSTT2 das im Gesamt-GST-Aktivitätsassay eingesetzte Substrat 1-Chloro-2,4-dinitrobenzene (Habig *et al.*, 1974) nicht abbaut (Pemble *et al.*, 1994; Tan *et al.*, 1996). Für die Modulation der GSTA-Isoformen, die auf niedrigerem Niveau exprimiert sind, war die Enzymaktivitätsmessung womöglich nicht sensitiv genug. Vor allem eine Induktion der hoch exprimierten GSTP1 wäre mit diesem Substrat erfassbar (Ebert *et al.*, 2001). Damit Änderungen auf Proteinebene zum Tragen kommen, könnten zudem längere Inkubationszeiten nötig sein (Ebert *et al.*, 2003).

Insgesamt zeigen diese Untersuchungen einen protektiven Einfluss von Butyrat und des Fermentationsüberstandes aus Inulin auf primäre, nicht-transformierte humane Kolonzellen. Insbesondere die Induktion der GST könnte dazu beitragen, die Zellen vor genotoxischen Stoffen zu schützen, da Xenobiotika so schneller konjugiert und ausgeschieden werden können.

4.4 Modulation von Enzymen der Stress-Abwehr durch Butyrat

Oxidativer Stress entsteht, wenn zelluläre Schutz- und Reparatursysteme aufgrund vermehrter Bildung von ROS überlastet sind. Endogen werden ROS in der Atmungskette, in der β-Oxidation von Fettsäuren, im mikrosomalen Cytochrom P450-Metabolismus von Xenobiotika oder während der Phagozytose gebildet. Unter normalen Bedingungen werden ROS durch SOD, Katalase oder Glutathion-peroxidase abgebaut (Hayes & McLellan, 1999).

In Untersuchungen zur Modulierbarkeit von Genen der zellulären Stress-Antwort konnte gezeigt werden, dass Stress-assoziierte Gene auch durch eine Butyrat-Vorbehandlung verändert werden (***Publikation IV***).

Laut Gen-Array-Analyse wurde die Expression von Katalase und Metallothionein 2A in primären Kolonzellen induziert. Insbesondere eine gesteigerte Aktivität der Katalase kann die Zellen vor H_2O_2 schützen. Die Senkung der H_2O_2-Exposition könnte die Wahrscheinlichkeit von DNA-Schäden und dadurch das Risiko für Mutationen senken (Mates & Sanchez-Jimenez, 2000). Metallothioneine sind Metall-bindende Proteine, die Metallionen, freie Radikale und aktivierte Xenobiotika abfangen (Pemble *et al.*, 1994; Coyle *et al.*, 2002). Diese Veränderungen könnten Zellen vor oxidativem und metabolischem Stress schützen.

Cyclooxygenasen sind für die Umwandlung von Arachidonsäure zu Prostaglandinen verantwortlich. Es existieren zwei Isoformen, die COX-1, welche konstitutiv exprimiert wird, und COX-2, deren Expression bei Entzündungsreaktionen induziert ist und die in Dickdarmtumoren überexprimiert wird. Ferner spielt COX-2 eine Rolle bei der Krebsprogression (Church *et al.*, 2004). Die Ergebnisse einer Studie mit Ratten zeigten, dass eine Fütterung mit Inulin die erhöhte Expression von COX-2 und der induzierbaren Stickstoffmonoxid-Synthase (iNOS) in Tumoren der Ratten senken konnte (Femia *et al.*, 2002). Da eine Überexpression dieser beiden Gene in Zusammenhang mit einer Resistenz gegenüber Apoptose, gesteigerter Proliferation, DNA-Schädigungen und oxidativem Stress steht (Miyanishi *et al.*, 2001), könnte die Reduktion der Expression derartiger Gene eine Erklärung für die verringerte Tumorbildung in den AOM-behandelten Tieren nach Präbiotikagabe liefern.

Im Gen-Array mit primären Kolonzellen wurde eine Reduktion der COX-2-Expression detektiert (***Publikation IV***). Dies könnte dazu beitragen, inflammatorische Prozesse zu reduzieren, welche mit erhöhter Proliferation im entzündeten Gewebe sowie oxidativem Stress einhergehen (Fournier & Gordon, 2000). Insgesamt stellt die Verringerung entzündlicher Reaktionen einen sinnvollen Ansatz der Chemoprotektion dar, da ein Zusammenhang zwischen Kolonkrebsentstehung und chronischer Inflammation besteht (Munkholm, 2003).

Im Gegensatz dazu wurde die Expression von SOD2 und Glutathionreduktase reduziert. Die SOD2 fängt Superoxidanionen ab, die zu H_2O_2 reduziert werden. Dieses Produkt ist für die Zelle kurzfristig weniger schädlich als Sauerstoffradikale bzw. ist sogar als Signalmolekül von Bedeutung (Rhee, 2006). Dabei wird GSH oxidiert, was durch die Glutathionreduktase wieder reduziert werden kann. Da die Expression von SOD2 in den untersuchten Spendern trotz Senkung weiterhin auf hohem Niveau lag, bleibt zu ermitteln, ob diese Senkung funktionelle Konsequenzen hat. Da andere Studien eine Senkung der Genotoxizität von H_2O_2 in humanen Kolonzellen zeigen konnten (Abrahamse *et al.*, 1999; Rosignoli *et al.*, 2001), könnte möglicherweise der Induktion von Katalase und Metallothionein 2A eine größere funktionelle Bedeutung zukommen.

Auch die Vorbehandlung der Zellen mit dem aus Inulin hergestellten Fermentationsüberstand, vermochte die Katalase-Expression zu steigern, wobei Butyrat in den für diese Versuchsreihe verwendeten Gewebeproben die stärkste Induktion verursachte (***Publikation V***).

Bei der Untersuchung der Genexpression von Katalase, SOD2 und COX-2 in primären Kolonzellen weiterer Spender mit real-time PCR wurde hinsichtlich der Modulierbarkeit durch Butyrat eine beträchtliche interindividuelle Variation deutlich (***Publikation IV***). In vier von sechs Spendern konnte eine Steigerung der Katalase-Expression gezeigt werden; in vier von sechs Spendern wurde COX-2 gesenkt und in vier von sechs Individuen senkte die Butyratbehandlung die SOD2-Expression.
Die divergierenden Effekte für die Katalase-Expression konnten auch nach Ermittlung des Genotyps für den Katalase-Promotor nicht erklärt werden. Die genetische Ausstattung, die Exposition gegenüber den in den Fäzes enthaltenen Noxen und die individuelle Zusammensetzung der Dickdarmflora der einzelnen Spender könnten zum einen Einfluss auf die Expressionshöhe der untersuchten Gene haben, zum anderen die Modulierbarkeit durch Butyrat beeinflussen. Festzuhalten bleibt, dass physiologische Konzentrationen an Butyrat in einigen Spendern chemoprotektive Veränderungen der Expression von Stress-assoziierten Genen hervorrufen können. Allerdings scheinen durch bislang nicht geklärte individuelle Einflussgrößen einige Spender mehr als andere von dieser Modulation profitieren zu können.

4.5 Vergleich der Genexpression in normalem und Tumorgewebe desselben Spenders

Aufgrund der hohen interindividuellen Variabilität insbesondere hinsichtlich der Ausprägung Butyrat-induzierter Genexpressionsveränderungen stellt der Vergleich von normalem Gewebe mit Tumorgewebe desselben Spenders einen wichtigen experimentellen Ansatz dar, um die Wirkungen von Butyrat direkt zwischen Zelltypen unterschiedlichen Transformationsgrades zu vergleichen (***Publikation VI***). *In vivo* wurde bereits mehrfach ein Vergleich der Genexpression in Kolonbiopsien aus normalem und Tumorgewebe vorgenommen (Sugiyama *et al.*, 2005; Chiu *et al.*, 2005). In den hier dargestellten vergleichenden Genexpressionsanalysen vier verschiedener Spender konnten einige Gene identifiziert werden, deren Expression sich signifikant zwischen normalen und Tumorzellen unterscheidet (GSTA-Isoformen und Glutathionperoxidase). Andererseits wurde für einige Gene kein Unterschied zwischen den verschiedenen Transformationsstadien gefunden, wie GSTP1 und COX-2, obwohl für beide Gene eine Überexpression in Kolontumoren beschrieben wurde (Fournier & Gordon, 2000; Miyanishi *et al.*, 2001). Von 192 erfassten Genen wurden 29 als signifikant differentiell exprimiert ermittelt. Davon waren 10 in

Tumorgewebe höher exprimiert als im normalen Gewebe, 19 dagegen waren im normalen Gewebe stärker exprimiert. Für diese Auswertungen ist es von Bedeutung, die Genexpression weiterer Spender zu erfassen bzw. mit Daten der Literatur zu vergleichen. Der Vergleich der Ergebnisse dieser vier Spender mit Gendatenbanken (Altenberg & Greulich, 2004) lieferte für fast 70 % der differentiell exprimierten Gene Übereinstimmungen.

Da bei nahezu jeder zweiten Person mit einer „Western Style Diet“ im Alter >70 Jahre Kolonadenome auftreten (Fodde *et al.*, 2001), können Darmfermentationsprodukte, wie Buttersäure, in zahlreichen Personen auf gesunde und entartete Zellen gleichzeitig wirken. Für Buttersäure wurde ein paradoxer Effekt auf normale und entartete Zellen beschrieben (Gibson *et al.*, 1999). Untersuchungen in normalen und Tumorzellen wurden bisher jedoch an Zellen unterschiedlicher Herkunft vorgenommen. Die detektierten Unterschiede bezüglich der Genexpression sind zum einen vom Transformationsgrad der Zellen abhängig, zum anderen von den Kultivierungsbedingungen der Zellen (Fenton & Hord, 2006).

Als Zielgene, die parallel in normalem und Tumorgewebe desselben Spenders auf ihre Modulierbarkeit durch Butyrat untersucht wurden, wurden Katalase und COX-2 als Vertreter Stress-assoziierter Gene ausgewählt und GSTP1 und GSTM2 als Zielgene der Biotransformation definiert. Für letztere wurde bereits eine Induzierbarkeit durch Butyrat in Tumorzellinien beschrieben (Ebert *et al.*, 2001; Ebert *et al.*, 2003). Katalase und COX-2 erwiesen sich in primären Kolonzellen als modulierbar (***Publikation IV***). Bislang standen Gewebe von drei Spendern für diesen Vergleich zur Verfügung. Aus diesen Daten lassen sich noch keine abschließenden Schlussfolgerungen ableiten. Die Modulierbarkeit scheint jedoch in stärkerem Maße von individuellen Einflussgrößen abzuhängen als von der Gewebeart.

4.6 Zusammenfassende Schlussfolgerungen

Aus den in dieser Arbeit durchgeführten Untersuchungen lassen sich folgende Schlussfolgerungen ableiten:

- Die Eisenquellen Hämoglobin und das oxidierte Produkt Hämin aus rotem Fleisch wirken über die Bildung von Radikalen genotoxisch in primären Kolonzellen. Eine hohe Aufnahme an rotem Fleisch könnte daher an der Initiation nicht-transformierter Zellen beteiligt sein.

- Aufgrund individuell verschiedener GST-Expressionsniveaus könnten Individuen mit geringeren Expressionshöhen durch eine höhere Exposition gegenüber karzinogenen Substraten der GST ein erhöhtes Risiko für DNA-Schäden haben.
- Mit der Verlängerung der Lebenszeit primärer Kolonzellen *ex vivo* wurde es möglich, modulierende Effekte von Nahrungsinhaltsstoffen an einem nicht-transformierten Zellmodell zu untersuchen.
- Obwohl nur geringe Mengen des applizierten Butyrats von den Zellen aufgenommen wurden, können GST auch in primären Kolonzellen durch das Darmfermentationsprodukt Butyrat induziert werden, wodurch die Entgiftungskapazität der Zellen gesteigert werden kann. Im Gegensatz zur Wirkung auf Tumorzellen sind physiologische Butyratkonzentrationen für primäre Kolonzellen nicht toxisch.
- Butyrat steigerte die Expression von Katalase in Zellen einiger Spender, wodurch diese besser vor H_2O_2 geschützt sind, und senkte die Expression von COX-2, was inflammatorische Prozesse verringern könnte.
- Komplexe Darmfermentationsprodukte aus Inulin können trotz geringer Butyratkonzentration in primären Kolonzellen die metabolische Aktivität steigern und Gene für Entgiftungsenzyme modulieren. Dies deutet zum einen auf trophische Effekte des Fermentationsproduktes hin, zum anderen wird durch die Modulation von Entgiftungsenzymen die Abwehrkapazität der Zellen erhöht. Der Fäzes-Kontrollüberstand veränderte auch die Genexpression, was auf weitere aktive bakterielle Fermentationsprodukte zurückzuführen ist, die jedoch noch weiterer Klärung bedürfen.
- Die Zielgene der Butyratwirkung in nicht-transformierten Kolonzellen sind mit chemoprotektiven Effekten assoziiert. Insbesondere eine längerfristige Steigerung der Aktivität von Entgiftungsenzymen könnte primäre Kolonzellen vor oxidativem Stress oder genotoxischen Substanzen schützen. Aufgrund starker interindividueller Variationen können einige Individuen mehr von diesen Effekten profitieren als andere.
- Ein ausreichender Verzehr von Ballaststoffen, wie z.B. Inulin, der zu wirkungsvollen Butyratkonzentrationen im Darmlumen führt, könnte über die hier dargestellten Mechanismen einen Beitrag zur Kolonkrebsprävention durch die Ernährung leisten. Ob jedoch diese *in vitro* gefundenen Effekte auch *in vivo* durch

eine Ernährung reich an Inulin (bzw. an Ballaststoffen) hervorgerufen werden, muss in weiterführenden Untersuchungen noch gezeigt werden.

4.7 Ausblick

Primäre, humane Kolonzellen stellen ein viel versprechendes Zellmodell dar, Mechanismen der primären Chemoprävention durch Darmfermentationsprodukte zu beleuchten. Dieses Zellmodell kann aber auch für weitere Substanzen, welche eine Rolle bei der Induktion oder Hemmung der Kolonkrebsentstehung spielen können, eingesetzt werden.

Es ist notwendig, die Zellkulturbedingungen weiter zu optimieren, um den physiologischen Bedingungen und Bedürfnissen der Zellen noch näher zu kommen. Um den Einfluss von Fermentationsprodukten auf Transkriptions- und Translationsebene zu erfassen und direkt vergleichen zu können, sollten in nachfolgenden Experimenten mehrere biologische Parameter für einen Spender gleichzeitig erfasst werden. Darüber hinaus ist es notwendig, die Anzahl der untersuchten Spender zu erhöhen, um trotz interindividueller Variationen Tendenzen ableiten zu können.

Im Vordergrund weiterer Experimente sollte die Erfassung von funktionellen Konsequenzen stehen, die beispielsweise als eine reduzierte Genotoxizität von Risikofaktoren für Kolonkrebs messbar wären.

Nach wie vor interessiert die Klärung der Frage, ob Ballaststoffe aufgrund der Bildung von Butyrat als Produkt der mikrobiellen Fermentation im Darm protektiv bzw. antikanzerogen wirken können und ob und inwiefern dies für normale und entartete Kolonzellen gleichermaßen gilt.

5 Zusammenfassung

Einleitung: Epidemiologischen Studien zufolge hat die Ernährung großen Einfluss auf die Kolonkrebsentstehung. Im Gegensatz zu schädlichen Inhaltsstoffen, wie z.B. Eisen aus rotem Fleisch, ist der Verzehr von Obst und Gemüse mit einer Senkung des Kolonkrebsrisikos assoziiert. Durch die damit verbundene Aufnahme von Ballaststoffen werden durch Bakterien im Dickdarm Fermentationsprodukte gebildet. Von diesen wird der kurzkettigen Fettsäure Butyrat eine chemopräventive Bedeutung zugesprochen. Es wurde gezeigt, dass Butyrat unterschiedliche Wirkungen auf normale und Tumorzellen hat. So wurde in Kolontumorzellen die Proliferation gehemmt, Apoptose induziert und Entgiftungsenzyme moduliert. Normale Zellen verwenden Butyrat als Energiequelle. In Tierstudien führte die Gabe von Inulin, einem präbiotischen Ballaststoff, aus welchem im Kolon Butyrat gebildet wird, zur Senkung einer chemisch induzierten Kolonkarzinogenese.

Zielstellung: Ziel dieser Arbeit bestand darin, Mechanismen der Chemoprävention durch Darmfermentationsprodukte in nicht-transformierten Kolonzellen zu untersuchen.

Methoden: Primäre Kolonzellen wurden aus humanem Dickdarmgewebe isoliert. Als mögliche Risikofaktoren wurden Hämoglobin und Hämin auf ihr genotoxisches Potential mittels Comet Assay untersucht. Durch eine Weiterentwicklung der Zellkulturtechnik wurden primäre Zellen bis zu zwölf Stunden mit Butyrat bzw. einem komplexen Fermentationsüberstand aus Inulin inkubiert, um den Einfluss auf die Vitalität und metabolische Aktivität zu ermitteln. RNA wurde isoliert, um mittels Gen-Arrays und real-time PCR den Einfluss auf die Genexpression zu untersuchen. Einen besonderen Schwerpunkt stellten GST dar, sowie Gene, die für antioxidative und inflammatorische Enzyme kodieren. In vergleichenden Untersuchungen wurden basale Genexpression und der Einfluss von Butyrat auf normale und Tumorzellen des gleichen Spenders untersucht. Für GST und Katalase wurden außerdem Enzymaktivitätsmessungen durchgeführt. Um interindividuelle Variationen der Expressionshöhe und Modulierbarkeit zu erklären, wurden vier GST- und ein Katalase-Promotor-Polymorphismus bestimmt.

Ergebnisse: Eisenverbindungen aus rotem Fleisch induzieren DNA-Strangbrüche in primären Kolonzellen. Darmfermentationsprodukte waren in physiologischen Konzentrationen in primären Kolonzellen nicht toxisch. Die Gen-Array Ergebnisse zeigten, dass nicht-transformierte Kolonzellen mit starken Schwankungsbreiten

Entgiftungsenzyme exprimieren. Neben der Verwendung als Energiequelle induzierte Butyrat GSTA-Isoformen und GSTT2. Trotz einer geringen Aufnahme des den Zellen zur Verfügung gestellten Butyrats, wurde in einigen Spendern Katalase induziert, SOD2 und COX-2 gesenkt. Auch der komplexe Fermentationsüberstand sowie der Fäzes-Kontrollüberstand beeinflussten die Genexpression. Zwischen den ermittelten Genotypen und interindividuellen Variationen konnte kein Zusammenhang gefunden werden.

Schlussfolgerung: Primäre, nicht-transformierte Kolonzellen stellen ein wichtiges Zellmodell dar, Mechanismen der Primärprävention zu untersuchen. Die Steigerung von Entgiftungssystemen durch Darmfermentationsprodukte könnte die Zellen vor genotoxischen Substanzen und oxidativem Stress schützen und so eine Möglichkeit der Darmkrebsprävention durch Ballaststoffe in der Ernährung darstellen.

5 Abstract

Introduction: Nutrition plays an important role in colon carcinogenesis. In contrast to risk factors like iron from red meat, the consumption of fruits and vegetables is associated with the prevention of colon cancer. This may be due to dietary fibres which are fermented by the gut flora to yield short chain fatty acids. In particular, butyrate has several chemopreventive properties. In tumour cells, it inhibits proliferation, induces apoptosis and increases detoxification enzymes, whereas in normal cells it is used as an energy source. Animal studies showed a reduction of chemically induced colon carcinogenesis after feeding the prebiotic inulin.

Aim: The aim of this work was to analyse mechanisms of chemoprevention by colonic fermentation products in non-transformed colon cells.

Methods: Primary colonocytes were isolated from human colon tissue specimens. The genotoxic potential of haemoglobin and haemin was studied in primary colon cells using the comet assay. After further development of a primary cell culture technique the primary cells were treated up to twelve hours with butyrate or a complex fermentation supernatant of inulin to elucidate effects on viability and metabolic activity. RNA was isolated to study gene expression using gene arrays and real-time PCR with special attention to GST, antioxidative and inflammatory genes. Basal gene expression and the impact of a butyrate treatment were compared between normal and tumour cells derived from the same donor. In cytosols, enzyme activities of GST and catalase were measured. To explain interindividual variations of

gene expression levels and modulation, four polymorphisms of GST and a catalase promotor polymorphism were determined.

Results: Iron from red meat induced DNA strand breaks in primary colon cells. Colonic fermentation supernatants in physiological concentrations were not toxic in primary cells. The gene array analysis revealed strong interindividual variations of expression levels of detoxification enzymes. Butyrate is not only an energy source but can also induce GSTA isoforms and GSTT2. Even only small amounts of butyrate were absorbed by the cells, catalase was induced in some donors of cells, and SOD2 and COX-2 were reduced. There was no correlation between the determined genotypes and gene expression variations.

Conclusions: Primary, non-transformed colonocytes represent an important cell model to study mechanisms of primary cancer prevention. The induction of detoxification enzymes by fermentation products may protect the cells from genotoxic compounds and from oxidative stress and may present a feasible mean to reduce colon cancer risk by dietary fibre.

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Publikationen

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe.
Die Arbeit wurde in keiner Form einer anderen Prüfungsbehörde vorgelegt und auch nicht veröffentlicht.

Jena, den 12.01.2007 Julia Sauer

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